THE ROLE OF TESTICULAR GERM CELL APOPTOSIS DURING EQUINE SPERMATOGENESIS

A Dissertation

by

NOAH LELAND HENINGER III

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2005

Major Subject: Physiology of Reproduction

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ABSTRACT

The Role of Testicular Germ Cell Apoptosis During

Equine Spermatogenesis. (December 2005)

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Apoptosis in testicular germ cells has been demonstrated in many species. Features of apoptosis reported in other species were used to confirm use of the TUNEL assay in stallion testes. Eight stallions with normal testicular size and semen quality were evaluated to determine the germ cell types and stages where apoptosis most commonly occurs. Mean numbers of TUNEL-positive germ cells per 100 Sertoli cell nuclei were highest in stages IV and V of the seminiferous epithelial cycle corresponding to meiotic divisions of primary spermatocytes and mitotic proliferation of B1 and B2 spermatogonia. Round and elongated spermatids were labeled less frequently by the TUNEL assay.

To examine the relationships between germ cell apoptotic rate and spermatogenic efficiency, seminal traits were assessed to classify stallions into normal or reduced quality semen groups. Apoptotic rates were higher for stages IV-VI and stage VIII seminiferous tubules in stallions with reduced semen quality. Daily sperm production (DSP) per gram and per testis were lower for stallions with reduced semen quality.

Regression analyses revealed negative linear relationships for germ cell apoptotic rate with DSP/g, DSP/testis, daily sperm output, progressively motile sperm and morphologically normal sperm in ejaculates. Mean circulating concentrations of inhibin were lower for stallions ejaculating reduced quality semen. Apoptotic rate was negatively correlated with concentrations of inhibin and estradiol-17β and positively correlated with concentrations of LH and FSH.

To study germ cell apoptosis and formation of the Sertoli cell barrier during the initiation of spermatogenesis, tubule development was classified based on lumen score. Formation of a seminiferous tubule lumen was consistent with events leading to development of a Sertoli cell barrier. A primary wave of apoptosis removed early differentiating germ cell types prior to the formation of a tubule lumen facilitating both the formation of a tubule lumen and a Sertoli cell barrier. A second wave of apoptosis occurred after the formation of a lumen but before seminiferous tubule cross-sections contained a full complement of germ cells.

In conclusion, apoptosis is an essential mechanism during normal spermatogenesis. Apoptosis also accounts for low numbers of normal sperm seen in the ejaculates of some stallions.

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Without my family, I could not be who I am today. To my wife Jennifer, thank you for your love, companionship, and support. Your sacrifices have allowed me to pursue my lifelong ambitions. When times get tough, I know we can always look to each other. Special recognition should also go to my father who purchased my first horse. The mare bucked me off and put me in the hospital with a concussion, but the event quite literally spurred onward my love of horses and led to my enthusiastic desire to understand the equine species. My father's words of wisdom regarding the event were, "If you would just stay on top, you won't have the problem", which now seems like a pretty good way to look at any situation life may have to offer. To my mother, my sister, and my grandparents, thank you for insisting that I accept nothing less than the best life has to offer. There are always good times in the past, the present and the future if you know where to look for them.

TABLE OF CONTENTS

Page

CHAPTER Page

LIST OF FIGURES

FIGURE Page

LIST OF TABLES

CHAPTER I

INTRODUCTION

Apoptosis (programmed cell death) is a well-defined physiological process that serves to eliminate damaged, diseased, or superfluous cells from various tissues of the body. Apoptosis in testicular germ cells has been demonstrated in many species, but little is known about apoptosis in spermatogenic cells of stallions (*Equus caballus*). The stallion accounts for 50% of the outcome of any attempted mating and so, fertility defects in a stallion can have serious consequences on success rates of any breeding program. Assessing a stallion's fertility can be a difficult process and may be confounded by fertility of the mares and quality of breeding management [1]. The reluctance of stallion owners to share fertility information for a given stallion, coupled with a reluctance to select for reproductive performance is also thought to contribute to relatively low fertility rates. Average fertility rates for light-breed stallions have been estimated to be as low as 52% based on seasonal pregnancy rates [2]. A recent study of 106 stallions recently retired from performance careers revealed that 43% failed to pass routine breeding soundness exams primarily due to ejaculation of insufficient numbers of normal sperm [3]. Understanding the physiological events leading to reproductive dysfunction in the stallion could improve foaling rates and aid in salvaging valuable genetics from subfertile stallions.

Studies in various mammalian species have shown that apoptosis is necessary for normal sperm production (spermatogenesis) in the testes [4,5]. Other experiments have

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This dissertation follows the style of Theriogenology.

also shown higher rates of apoptosis in individuals with clinically identified idiopathic subfertility/infertility [6,7]. Experiments in this dissertation were designed to develop a better understanding of the process of apoptosis in stallion testes, while exploring the relationships for level of apoptosis (high vs. low), with semen quality and measurable reproductive parameters (listed below). Unsatisfactory semen quality in stallions contributes to low pregnancy rates and results in substantial financial loss in the equine industry. Cellular and molecular factors identified in these experiments that may contribute to reduced semen quality will be the target of future research aimed at proper diagnosis of fertility problems in conjunction with improvement of semen quality and stallion fertility.

1. Objectives

1.1. Experiment I: Germ cell apoptosis in the testis of normal stallions

1. Validation of methodology to evaluate apoptosis both quantitatively and qualitatively in stallion testes including:
    a. Terminal dUTP Nick End Labeling (TUNEL) Assay,
    b. Evaluation of apoptosis in an untreated vs. treated (heat/GnRH-agonist) testis from a single stallion,
    c. Confirm low molecular weight (mw) DNA fragmentation using the ladder assay, and
    d. Ultrastructural analysis of apoptotic germ cells.

2. Develop methodology and criteria to stage the equine seminiferous epithelium using a novel combination of Periodic-Acid-Schiff (PAS) and Toluidine blue counter stain.
3. Identify the stages and germ cell types where apoptosis commonly occurs in mature stallions with normal semen quality.
4. Quantify germ cell apoptotic rates (expressed as the number of apoptotic germ cells per 100 Sertoli cell nuclei) in mature stallions with normal semen quality.

1.2. Experiment II: Apoptosis in the testes of stallions with reduced semen quality

1. Use the TUNEL assay to quantify normal semen quality for comparison to stallions with reduced semen quality.
2. Quantify the testicular and endocrine factors (daily sperm production per gram parenchyma (DSP/g), daily sperm output (DSO), sperm motility, sperm morphology, and hormonal concentrations (FSH, LH, inhibin, estradiol and testosterone) for comparison to the rate of germ cell apoptosis. A statistical approach was used to identify factors that relate to increased or decreased rates of apoptosis for future research and therapeutic treatment strategies designed to modulate germ cell apoptotic rates.

1.3. Experiment III: Apoptosis during the initiation of equine spermatogenesis

1. Provide a redefined classification scheme describing testicular development in terms of events leading to the formation of a seminiferous tubule lumen

(lumen score) and the presence of advanced germ cell types in pubertal stallions.

2. Exploit the organized, regional pattern of spermatogenic development in the pubertal stallion to determine whether apoptosis of germ cells occurs at higher rates during specific steps of seminiferous tubule development.
3. Examine the development of the Sertoli cell barrier as it relates to events leading to the formation of a tubule lumen and germ cell apoptotic rates
4. Develop schematic, grid-based maps of testicular and seminiferous tubule development from histological cross-sections of pubertal stallion testes (ages 9-14 months).

CHAPTER II

REVIEW OF THE LITERATURE

1. Spermatogenesis in the Stallion

During gestation in the early embryo, primordial germ cells migrate to the genital ridge and colonize the undifferentiated fetal gonads. Decent of the testes generally occurs between 30 days before and 10 days after birth [8]. At birth, the stallion testis contains only indifferent supporting cells, and gonocytes [9]. Until puberty, these cells remain in closed, rudimentary seminiferous tubules (dark parenchyma) until testes development and systemic pubertal changes begin to occur. At puberty, gonocytes differentiate spermatogonia and the dark indifferent supporting cells differentiate into Sertoli cells [10]. A gross-section through a developing testis from a pubertal stallion has light testicular parenchyma with expanding seminiferous tubules in the center, surrounded by dark testicular parenchyma in the periphery, where tubules are less developed [10]. Spermatogonia (A_S or A_0) then differentiate into the various morphologic forms (A1-A4 and B1-B2) of spermatogonia giving rise to more advanced generations of germ cells (primary spermatocytes and spermatids) as pubertal development progresses. Seminiferous tubules begin to form a tubule lumen by unidirectional fluid secretion forming small vacuoles in the area of the developing tubule lumen [11,12] forming what will be ducts to transport mature spermatozoa from their site of production in the seminiferous tubule to the epididymis.

Spermatogenesis in the mature stallion is the sum of all cell divisions that result in the formation of spermatozoa from a single spermatogonia [9]. Spermatogenesis can

be subdivided into spermatocytogenesis, meiosis, and spermiogenesis [10]. Spermatocytogenesis encompasses mitotic proliferations of spermatogonia generating spermatocytes for meiotic division. Two divisions occur as part of meiosis. In the first division (reduction division) DNA is synthesized during a protracted prophase. Thin filaments of chromatin (unevenly distributed such that one side of the nucleus appears light [10]) with each chromosome composed of sister chromatids (leptotene), followed by the pairing of homologous chromosomes (zygotene) and exchange of genetic material (pachytene) [13]. The four chromatids of each homologous pair become tightly associated. Crossing-over during this association permits an exchange of genes among chromatids resulting in a recombination of genes and genetically dissimilar daughter cells. In the diplotene stage, areas of genetic exchange are called chiasmata [13]. During diakinesis, the chromosomes condense, and then line up in the equatorial region (metaphase). Homologous chromosomes then move to opposite sides (anaphase) and separate by cytokinesis (telophase) producing two haploid secondary spermatocytes with double-stranded, unpaired chromosomes (sister chromatids) [13]. The second nuclear division (equatorial division) involves no replication of DNA. Chromosomes (each composed of two chromatids) line up at the equatorial plane and chromatids (from each chromosome) separate resulting in the production of haploid round spermatids containing half the original number of chromosomes. Each primary spermatocyte therefore produces four spermatids. Spermiogenesis is the morphologic differentiation of spermatids to spermatozoa [10] and begins with newly formed Sa round spermatids. These spermatids differentiate into Sb1 (acquisition of a tail) and Sb2 (irregular shaped

nucleus) spermatids and move closer to the lumen of the seminiferous tubule. Elongation and condensation of the spermatids occurs (Sc Sd1 and Sd2), DNA synthesis decreases, and histones are replaced with transition proteins and finally protamines which facilitate compaction of DNA [14] and nuclear remodeling. Excess cytoplasm is removed from the elongated spermatids by residual bodies that are phagocytosed by apical regions of the Sertoli cell [15]. Mature elongated spermatozoa are released into the lumen of the seminiferous tubule spermiation.

The stage of seminiferous epithelium is defined as man-made divisions of naturally occurring cellular associations in a given region of a seminiferous tubule [10]. The morphologic classification system has served as the foundation for the study of spermatogenesis in most species. Acrosomal changes associated with spermatid development [16] have been found to be some of the most accurate characteristics by which stages could be divided. However, because spermatogenesis is a continuous biological process, tubules are often in transition between two stages, or contain more than one stage within a given cross-section. Thus, consistent and precise recognition of the stages requires recognition of slight changes in morphology, thinking in three dimensions and understanding that all observations occur over time.

For the stallion, the total length of spermatogenesis is equal to about 57 days [10]. Spermatocytogenesis, meiosis, spermiogenesis are approximately 19.4, 19.4, and 18.6 days respectively. It takes approximately 4.7 cycles of the seminiferous epithelium for one spermatogonia to complete spermatogenesis. For the stallion, stages of the seminiferous epithelium may be found in a sequential order along the length

seminiferous tubule with spermiation occurring every 12.2 days. The wave of seminiferous epithelium is postulated to aid in maintaining a constant supply of sperm, reducing competition for nutrients, and reducing tubular congestion from high numbers of spermatozoa. The frequency with which a seminiferous tubule (stage) occurs in a given histological section differs between species. For the stallion the relative frequencies for the eight designated stages are approximately 16.9, 14.9, 3.2, 15.8, 7.4 13.5, 12.6, and 15.7% with the lower frequencies corresponding to shorter cycle durations of 2.1, 1.8, 0.4, 1.9, 0.9, 1.6, 1.5, and 1.9 days.

2. Sertoli Cell Function

Sertoli cells are critical for germ cell development during spermatogenesis. Germ cells cannot develop in absence of Sertoli cells, and the role of Sertoli cells at the molecular level has only begun to be characterized. A range of functions have been proposed for the Sertoli cells and includes maintaining an optimal environment for germ cell development and remodeling, removal of waste products and toxic metabolites, maintenance of the blood testis barrier, movement of germ cells from the basal to the adluminal compartment and spermiation, phagocytosis of germ cells and residual bodies, regulation of germ cell numbers, and cellular communication (endocrine and paracrine) [10]. Sertoli cells junctions form a complex matrix of proteins, the nature of which is often underestimated (for a review see [17]). Proteins most often found in the junctions joining the Sertoli cell consist of *zonula occludins*, *zonula adherins*, and desmosome-like proteins [18]. These junctional proteins are attached to cytoskeletal elements in the

Sertoli cell cytoplasm such as actin, myosin, and vimentin [18]. Most junctional proteins do not appear until puberty [18] suggesting that a barrier is not necessary until the initiation of spermatogenesis . Once formed however, the barrier is an extremely resistant and highly selective.

The creation of a specialized network of junctional proteins between Sertoli cells is generally referred to as the blood-testis-barrier. The barrier as the name implies facilitates the sequestration of advanced germ cells from blood and its serological components. This barrier is thought to be composed of endothelial cell junctions, junctions between peritubular myoid cells, a non-cellular component consisting of multiple layers (basal lamina surrounding each seminiferous tubule) and Sertoli cell junctional complexes. The Sertoli-Sertoli tight junctions (the Sertoli cell barrier) prevent the entry of large molecules (i.e. proteins) from entering the tubule via the paracellular route. The function of such a specialized barrier is thought to create a specialized environment that is essential for germ cells to develop [18]. This broad statement implies that certain factors appear to be crucial for germ cell development that Sertoli cells provide. Likely candidates include growth factors and nutrient substrates [19], as well as hormones [20]. Sertoli cells may also protect both germ cells, and the general body system from metabolic and gaseous waste processes of meiosis. Such specialized conditions necessary for germ-cell meiosis may generate or even require potentially toxic metabolites or adverse gaseous and nutrient conditions [18] that are supplied to germ cells, or degraded by Sertoli cells before they enter the systemic circulation.

The Sertoli cell may also provide a means for sequestration of germ cells from the immune system. Since the testis generates non-self antigens, advanced germ cells must be sequestered away from circulating immunoglobins and conversely prevent antigens from meiotic germ cells from entering the systemic circulation [18]. However, some reports support the existence of autoantigens on the surface of spermatogonia residing within the basal compartment [21], thereby demonstrating the importance of other factors in creating specialized conditions for normal gem cell development. Research has shown that the Sertoli cell and/or germ cells produce local immune suppressing factors (i.e. FasL) that may serve as a mechanism to reduce the number of leukocytic infiltration into the testicular parenchyma. Other components that are postulated to contribute; the slightly lower temperature of the testis and higher local concentrations of steroids, both provide nonspecific immune suppression in the local environment of the testis [21] and suppression of leukocyte activation. Collectively, a combination of all factors provide a plausible explanation for immunogenic exclusion. However, if the barrier is disrupted pathologically (i.e. orchitis/trauma resulting in TNFα secretion and inflammation [22]), an irreversible infertility can result by the opsonization and destruction of germ cells caused by anti-sperm antibodies.

3. General Apoptosis

Apoptosis, also referred to as programmed cell death, comes from the greek translation of the words “apo” meaning away from and “ptosis” meaning to fall. Apoptosis is a well-defined, physiological process that eliminates individual cells from

mammalian tissues [23]. Specific signals (ligand mediated or endogenous) initiate a highly regulated, gene-directed response of endogenous, enzymatic disassembly of organelles leading to cell ablation. The primary classical functions for apoptosis are the removal of cells during mitosis and meiosis (cell division), cells infected with a virus (disease), or cells that have lost control of their regulatory components (cancer). Apoptosis is known to play essential roles in reducing cell numbers during embryogenesis, central nervous system development, immune system development and function, response to irreversible genetic damage, organ involution, and tumor biology. Studies that have attempted to inhibit apoptosis (pharmacological or genetic knockouts) during crucial points of development, lead to severe defects, organ dysfunction and/or death of the individual (for a review see [24]).

Identification of specific factors responsible for initiation of the apoptotic cascade, and the order in which they occur depend heavily on the tissue, cell type, and environmental stimuli involved. Two apparent pathways (intrinsic and extrinsic) have been suggested to exist. Early intrinsic pathways potentially involved in initiating the apoptotic cascade are phosphatidylserine exposure [25] and cytochrome c release [26,27]. Initiation of the apoptotic signal transduction cascade by extrinsic factors (Fas-L, TNFα) begins with the ligand binding to the cellular membrane bound receptor. The ligand/receptor complex associates with specific death domains (80 amino acid intracellular sequence which are responsible for propagation of the apoptotic signal) activate various procaspase molecules, which in turn activate caspases. Caspases do not directly degrade DNA. They are proteases that degrade cellular proteins and act in

cascade fashion to activate each other by similar proteolytic cleavage. Caspases specifically cleave at the carboxy terminus of aspartate residues in target structural proteins called lamins [28]. Lamins are intricately associated with the nuclear matrix and are essential for the structural support of the nucleus. Once the lamins have been degraded by the various caspases, the cell nucleus undergoes the characteristic morphological changes associated with apoptosis such as nuclear margination and condensation of chromatin. Activation of endogenous endonucleases within the nucleus cleaves DNA at exposed internucleosomal regions yielding fragments in multiples of approximately 180 base pairs [29]. This single characteristic of apoptosis allows for highly specific identification of apoptotic cells and is considered to be a hallmark of apoptotic cells. Labeled 3'OH ends of this fragmented DNA may be extracted and separated on a gel for observation of a distinct "ladder" pattern [30-32] or observed *in situ* by the TUNEL assay (for a review see [33]).

Apoptosis differs from other processes of cellular demise (i.e., necrosis) in that apoptosis requires ATP to drive its biochemical pathways [34]. Rates of apoptosis vary depending on the tissue, cell type, and species being described. This suggests that cells inherently vary in their susceptibility to apoptotic stimulus. Several ATP dependant molecular pathways exist that allow cells to either initiate, or avoid apoptosis. Cell death is most likely a balance of intracellular pro-apoptotic (bax, bad, and bcl-X_S) and anti-apoptotic gene products (bcl-2, bcl-X_L, and bcl-w) and cells may respond either way, given the appropriate stimulus. Activation of these pathways from within the cell allows for the plasma membrane to remain intact and prevents intracellular antigens

from entering interstitial space that would otherwise trigger migrating leukocytes to initiate an extensive immunological response affecting the entire tissue rather than a single cell [24]. This factor becomes an extremely important detail when considering immune privilege during spermatogenesis in the testis.

The tissue dynamics of apoptotic cell death are an important concept since the death of a single cell does little to change the structure and function of an organism. Cell growth and cell death rates must be closely matched to maintain a stable population of cells. A low rate of cellular growth and death is expected in stable tissues such as the brain. This may be diametrically opposed to tissues like the testis where a high turnover of cells is compensated by elevated levels of cell growth and death. The entire process of apoptosis on a single cell level is thought to be extremely rapid. Previous work has shown that the time from initiation of plasma membrane blebbing to complete cellular destruction is approximately 34 minutes [35], but the time-course varies depending on the tissue involved and cell type. Final stages of apoptosis result in segmentation of remaining cellular components (i.e. DNA, RNA, and proteins) into apoptotic bodies [36]. Components from apoptotic cells may be recycled by phagocytes (i.e. Sertoli cells) for the growth and maintenance of both new and existing tissues [37] although further work remains to determine distribution of the recycled components.

4. Germ Cell Apoptosis in the Normal Testis

Normal spermatogenesis is a relatively inefficient process, resulting in the estimated loss of 25-75% of the potential number of mature spermatozoa produced in the

adult testis depending on species [38,39]. Failure to reach potential production levels results from an overproduction of early germ cell types [40] beyond that which can be sustained by supporting Sertoli cells. This insures maximum sperm production potential, and likely aids in the selection/production of genetically normal sperm, but results in a large number of cells that never achieve maturity. Apoptosis has been implicated in the testis as an essential mechanism for removing these developing germ cells from the seminiferous epithelium [5,41,42], and has been described in the testes of all mammalian species studied [43-48]. It has been suggested that normal testicular function (sperm production) may be altered/adjusted by changing either 1) the ability of a germ cell to proliferate without encountering cell death and/or 2) the timing of cell death during development, with respect to exponential division [49]. Cell death early in spermatogenesis would logically have a more profound effect on total cell numbers. Increased germ cell degeneration due to seasonal variation in sperm production has been shown for the stallion [50] as well as other mammals exhibiting seasonal reproduction [51-53].

The stage of seminiferous epithelium reflects multiple germ cell progeny at different points of development and differentiation. Cellular relationships between germ cell progenies permit classification of the equine seminiferous epithelium into eight different stages with spermiation marking the end of stage VIII [15,54]. During normal spermatogenesis, the stages which most often have elevated levels of apoptosis generally correspond to stages in which mitotic and meiotic peaks of germ cell development occur

[49]. This would suggest that germ cell types are more susceptible to death at specific times during spermatogenic differentiation.

5. Germ Cell Apoptosis and Idiopathic Subfertility

Apoptotic cell death has been identified as a factor in many cases of male infertility/subfertility. Male fertility issues may be due to disease, management or idiopathy. Apoptosis has been linked both directly and indirectly to various testicular pathologic conditions such as germ cell tumors [55], cryptorchidism [56], testicular torsion [57,58], and hypospermatogenesis [59,60]. Apoptosis in the testes of patients with fertility defects may be due to increased levels of "inappropriate" apoptosis (i.e. altered gene expression or environmental stimuli), or decreased levels of appropriate apoptosis [6] (i.e. cancer, disruption of the Sertoli cell barrier) in either germ cells, or their supporting cells.

One of the primary difficulties in studying events leading to clinical male infertility/subfertility is identification of the initiating lesion. One observed pathologic phenotype (i.e. testicular degeneration) can have any number of causes and confounding factors (age, breed and environment) which ultimately make arriving at a correct diagnosis difficult, and treatment frequently impossible. To control for this variability, experiments have attempted to manipulate conditions to induce germ cell apoptosis, so that molecular triggers and pathways of germ cell apoptosis may be derived and subjected to further research in hopes of rescuing germ cells when exposed to an applied stressor. To date, several published models exist for studying induced pathological states

such as ischemia-reperfusion [61,62], experimental cryptorchidism [63-66], scrotal/testicular heating [67,68], hypophysectomy [69], treatment with GnRH agonists (pituitary gonadotroph downregulation)[70,71], drug treatment [72-74], radiation [75,76], and exposure to environmental toxicants [76-80]. Outcomes vary depending on the treatment, but generally result in accelerated levels of apoptosis in a particular spermatogenic stage or germ cell type. However, when reviewing the literature, careful consideration must be given to the induced experimental conditions which may not reflect what occurs spontaneously within a given individual.

A diagnosis of testicular degeneration in the stallion may be based on a decrease in quality of ejaculates (low numbers of normal, motile sperm), a decrease in testicular size (which may or may not occur), and attenuation of circulating hormone concentrations. Previous studies have found that stallions with decreasing fertility generally have low estradiol and inhibin concentrations, and a concomitant increase in FSH [81-84]. A previous study noted two distinct times of increased germ cell degeneration during spermatogenesis in low sperm producing stallions [85]. Losses in low sperm producing stallions occurred early in prophase of meiosis and during spermiogenesis which, at least in part, accounted for decreased levels of sperm production and a decreased germ cell:Sertoli cell ratio. Since germ cell degeneration (quantified by histomorphometric quantification of potential germ cell numbers) is related to apoptosis (quantification of cells with biochemical and/or morphological hallmarks of apoptosis), apoptosis is a likely candidate for the removal of these germ cells from the seminiferous epithelium during periods of decreased sperm production.

6. Apoptosis and the Initiation of Spermatogenesis

Establishment of spermatogenesis requires a long period of time in domestic animals and even longer in humans. Initiation of spermatogenesis begins with the conversion of gonocytes into spermatogonia, which generally occurs randomly throughout the mammalian testis in most species [86] with a corresponding uniformity of parenchymal coloration or shading [87]. In the stallion, however, development of spermatogenesis evolves progressively from the inner toward the outer region of the testis [86,88,89] as the inactive dark tissue gives way to spermatogenically active light tissue [87].

Gross observation of a section through a developing testis from 1 to 2-year-old stallions revealed light testicular parenchyma with expansion of seminiferous tubules in the center and dark tissue on the peripheral regions where tubules were less developed [87]. This pattern of development suggests that a local control mechanism for the initiation of spermatogenesis [90]. The differences in gross color have been attributed to an abundance of fetal Leydig cells and large macrophages in the interstitium of the dark (immature) parenchyma. Advancing pubertal development leads to increased tubular volume density of germ cells and tubular lumen formation, as well as a decreased density of macrophages and fetal Leydig cells in the light tissue [87].

In the horse, gonocytes are present in the sex cords of the developing fetus. They persist in the male sex cords until just before puberty, at which time they divide to produce spermatogonia. Spermatogonia differentiate to produce spermatocytes by 12 months and round spermatids by 16 months of age. Complete spermatogenesis with a

full complement of germ cells is generally observed in most tubules by 24 to 36 months of age [90]. However, the timing of the appearance of specific germ cells during puberty depends heavily on region of the testis sampled, season, and stallion-to-stallion variation.

In horses, as in other species, large numbers of degenerating germ cells are can be detected during the initiation of spermatogenesis. An early physiological apoptotic wave, which occurs among germ cells during the first division and differentiation, has been shown to be necessary for the development of normal, mature spermatogenesis in other species [5,42,91]. The significance of the apoptotic wave during the first round of spermatogenesis has not been elucidated in detail. Studies in mice that inhibit apoptosis by knocking out key apoptotic genes have resulted in accumulation of spermatogonia and spermatocytes and subsequent infertility [41,42]. It is thought that apoptosis serves to remove germ cells from the seminiferous epithelium to facilitate the formation of the blood-testis barrier.

CHAPTER III

GERM CELL APOPTOSIS IN THE TESTES OF NORMAL STALLIONS*

1. Overview

Apoptosis in testicular germ cells has been demonstrated in many mammalian species. However, little is known about the stallion (*Equus caballus*) and rates of apoptosis during spermatogenesis. Morphological and biochemical features of apoptosis reported in other species were used to confirm that the TUNEL (TdT-mediated dUTP Nick end Labeling) assay is an acceptable method for identification and quantification of apoptotic germ cells in histological tissue sections from stallion testes. Seminiferous tubules from eight stallions with normal testicular size and semen quality were evaluated according to stage of seminiferous epithelium to determine the germ cell types and stages where apoptosis most commonly occurs. Spermatogonia and spermatocytes were the most common germ cell types labeled by the TUNEL assay. A low rate of round and elongated spermatids were labeled by the TUNEL assay. Mean numbers of TUNEL-positive germ cells per 100 Sertoli cell nuclei were highest in stages IV (15.5 ± 1.0) and V (13.5 ± 1.1) of the seminiferous epithelial cycle ($P < 0.001$). An intermediate level of apoptosis was detected in stage VI ($P < .02$). These stages (IV to VI) correspond to meiotic divisions of primary spermatocytes and mitotic proliferation of B1 and B2 spermatogonia. Establishing basal levels of germ cell apoptosis is a critical step towards

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understanding fertility and the role of apoptosis in regulating germ cell numbers during spermatogenesis.

2. Introduction

Apoptosis, also referred to as programmed cell death, is a well-defined, physiological process that eliminates individual cells from mammalian tissues [23]. Specific signals (ligand mediated or endogenous) initiate a highly regulated, gene-directed response of endogenous, enzymatic disassembly of organelles leading to cell ablation. Apoptosis differs from other processes of cellular elimination (i.e., necrosis) in that apoptosis requires ATP to drive its biochemical pathways [34]. One of the most distinguishing biochemical hallmarks of apoptotic cells is the attack of endogenous endonucleases on inter-nucleosomal regions of DNA. Enzymatic cleavage at these sites leads to DNA fragments in multiples of approximately 180 base pairs [29]. Labeled 3'OH ends of fragmented DNA may be extracted and separated on a gel for observation of a distinct "ladder" pattern [30-32] or observed in situ by the TUNEL assay (for a review see [33]).

Normal spermatogenesis is a relatively inefficient process, resulting in the estimated loss of 25 to 75% of the potential number of mature spermatozoa produced in the adult testis [38,39]. The lack of reaching potential production levels has been shown to result from an overproduction of early germ cell types [40], beyond that which can be sustained by supporting Sertoli cells. This insures maximum sperm production potential. Apoptosis has been implicated in the testis as an essential mechanism for removal of

developing germ cells from the seminiferous epithelium [5,41,42], and has been described in the testes of numerous mammalian species [43-48]. Apoptosis of germ cells in the stallion has not been reported.

The stage of seminiferous epithelium reflects multiple germ cell progeny at different developmental points of differentiation. During normal spermatogenesis, the stages which most often have elevated levels of apoptosis generally correspond to stages in which mitotic and meiotic peaks of germ cell development occur [49] and suggests that germ cell types are more susceptible to death at specific time points during differentiation. Cellular relationships among germ cell progenies in the equine permit classification of the seminiferous epithelium into eight different stages with spermiation marking the end of stage VIII [15,54].

The purposes of this study were to 1) confirm that the TUNEL assay (ApopTag® Serologicals Inc.) is a reliable method for labeling and quantifying apoptotic germ cells in fixed equine testicular parenchyma; and 2) investigate the hypothesis that the rate of apoptotic germ cell death differs between stages of seminiferous epithelium in mature, reproductively normal stallions. Examining germ cell death according to stage of seminiferous epithelium will reveal germ cell types which typically undergo apoptosis and provide a greater understanding of the mechanism and timing of germ cell apoptosis during spermatogenesis in the stallion

3. Materials and Methods

3.1. Confirmation of TUNEL labeling

To confirm that the TUNEL assay accurately labeled apoptotic germ cells in equine testis, a high-level of germ cell death was induced by a treatment exploiting the effects of heat (scrotal edema resulting from unilateral surgical orchiectomy) and pituitary gonadotropin down-regulation, which have both separately been shown to increase germ cell losses during spermatogenesis [71,92-94]. One 4-yr-old Thoroughbred stallion with normal testes size and semen quality was selected for orchiectomy under general anesthesia during the breeding season (August). One (left) testis was surgically removed to serve as a control prior to treatment. Post-surgical swelling of the scrotum was expected to induce heat-related testicular germ cell loss. Immediately following recovery from anesthesia, four 2.1 mg controlled-release deslorelin implants (Ovuplant^{TM}, Fort Dodge, IA) were placed subcutaneously in the stallion's neck. Deslorelin implants have been previously shown to induce pituitary gonadotropin down regulation in the stallion [95]. The remaining testis was removed 10 days post-treatment when the germ cell apoptotic rate was expected to be high. The following laboratory procedures were performed on testis samples from the control and treated testes:

1) Quantitative analysis comparing the number of TUNEL-positive germ cells per 100 Sertoli cell nuclei in the left (untreated) testis vs. the right (treated) testis.

2) An evaluation of 100 TUNEL-positive germ cells in unlabelled 3 μm serial sections to determine the specificity of the assay to appropriately label pyknotic germ cell nuclei.

3) Analysis of low molecular weight DNA fragmentation using a “ladder assay” on testicular parenchyma from the left (untreated) testis and right (treated) testis. DNA from normal and serum starved Jurkat cell culture served as negative and positive controls, respectively.

4) Ultrastructural analysis of germ cells to ensure germ cells exhibited morphological characteristics consistent with those reported in other species.

3.2. TUNEL immunohistochemistry

Immunohistochemical apoptotic detection (TUNEL assay) was performed on paraformaldehyde fixed 3 μm paraffin sections mounted on Superfrost plus® glass slides using an Apoptag® peroxidase detection kit S7100 (TUNEL) (Intergenco, Purchase, NY, USA) according to manufacturer’s instructions. After labeling, slides were stained with Periodic Acid-Schiff (PAS) stain for 10 min and counterstained with toluidine blue (1:100) for 1 min, and a cover slip was applied using Permount mounting media. Commercially available, unstained rat regressing mammary slides were used as positive controls (Intergenco, Purchase, NY, USA). To serve as a negative control, the Terminal deoxynucleotidyl Transferase (TdT) enzyme was omitted.

3.3. DNA ladder assay

DNA was isolated and analyzed from snap-frozen testicular parenchyma or cultured Jurkat cells (see below) according to a modification of the protocol by Billig et al [96]. Briefly, the labeling reaction was performed on DNA from treatment samples

(serum- deprived Jurkat cells, and right heat/GnRH-agonist treated stallion testis) and non-treated control DNA (healthy Jurkat cells and untreated left stallion testis). Incorporation of radioactive nucleotides (1μL 32^{P}dATP) onto the free 3'OH ends of fragmented DNA was accomplished using 5U Klenow enzyme. Unincorporated radioactive nucleotides were removed using a Micro bio-spin® P-30 Tris chromatography column (Bio-Rad laboratories). Labeled DNA (1μg) was fractionated through a 2% agarose gel (6.5 V/cm). Gel images were captured using phospho-imaging technology (Typhoon 8600 Phosphoimager, Amersham Biosciences, NJ) and processed in Adobe Photoshop® 5.0 (Adobe Systems, Seattle, WA).

3.3.1. Jurkat cell culture

A Jurkat cell line human T-cell leukemia clone (E6-1), was cultured according to Weiss et al [97] to provide control DNA for use with the ladder assay. Briefly, the Jurkat cell culture was maintained in logarithmic growth phase in RPMI media (Irvine-Scientific) with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, penicillin (100 units/ml) streptomycin (100 μg/ml), and 50 μM 2-mercaptoethanol. Cells were cultured in 5% CO_2/95% humidified air at 37°C at a density of 10^6 cells per ml. Serum deprivation has been previously shown to induce apoptosis in the Jurkat cell line [98]. Jurkat cells were cultured with serum or in serum-deprived conditions for 24 hr to serve as positive and negative controls. Cells were suspended in a hypotonic lysis buffer solution for DNA extraction.

3.4. Electron microscopy

Testicular parenchymal samples were processed for transmission electron microscopy according to Johnson et al [99]. Briefly, 1 mm^3 cubes were immersion-fixed in 2% glutaraldehyde for 24 hr and transferred to 100 mM cacodylate buffer. Samples were further fixed in osmium and embedded in Epon 812. Samples were sectioned at 60 nm and impregnated with uranyl acetate and lead citrate for ultrastructural detection of apoptotic features (ie. chromatin condensation, membrane blebbing) by transmission electron microscopy (Zeiss TEM 10CA).

3.5. Quantification of germ cell apoptotic rates in reproductively normal stallions

3.5.1. Animals

To establish germ cell apoptotic rates in reproductively normal stallions, the left and right testes were surgically removed under general anesthesia from eight mature (age 4 to17 yr), light-breed stallions with normal testes size and semen quality during the breeding season (June). Testicular parenchyma was processed for the TUNEL assay as described above.

3.5.2. Histological analysis

To quantify germ cell apoptotic rates, seminiferous tubules were evaluated using brightfield-illumination under oil immersion at 630X magnification (Axioplan2 Photomicroscope, Zeiss Inc., New York, NY). One hundred round cross-sections of seminiferous tubules from each testis were classified by stage of the seminiferous

epithelium (I-VIII) according to Swierstra et al [54] and Johnson et al [15]. A Periodic Acid –Schiff (PAS) staining technique proposed by Clermont and Leblond [16] was used for accurate assessment of acrosomal morphology. A detailed photomicrographic staging map was constructed to facilitate identification of germ cells in paraffin sections (Figure 1). Only round tubule cross-sections containing at least 75% of a respective stage were evaluated and relative frequencies for each stage of seminiferous epithelium were recorded. The apoptotic rate for each stage was expressed as the number of TUNEL-positive germ cells per 100 Sertoli cell nuclei to compensate for histological shrinkage and differences in tubule diameter. Stage of seminiferous epithelium, position of germ cells in the seminiferous epithelium and nuclear diameter were used as criteria for identification of degenerating germ cell types.

3.5.3. Statistics

Data obtained for the mean number of Sertoli cell nuclei per seminiferous tubule and number of TUNEL-positive germ cells per 100 Sertoli cell nuclei were analyzed by one-way analysis of variance using a 5% level of significance, with means separated post hoc using Tukey's Honest Significant Difference (HSD) (SAS® statistical software; Cary, NC 2000).

4. Results

4.1. Confirmation of TUNEL labeling

A three-fold increase in the number of TUNEL-positive germ cells per 100 Sertoli cell nuclei was detected in the right heat/GnRH-agonist treated testis (24.1) when compared to the left untreated testis (7.9) of the 4-yr-old Thoroughbred stallion. Decreases in seminiferous tubule diameter and height of the seminiferous epithelium were observed in the right testis of the treated stallion. Pyknotic nuclei were evident in 3 μm adjacent sections for 97 of 100 Apoptag®- labeled germ cells (Figure 2). Rare instances were noted where pyknotic nuclei were not stained by the TUNEL assay. Several instances of Sertoli cell apoptosis were noted in the treated testis, but not in the untreated testis of this stallion.

4.2. DNA ladder assay

Isotopic end-labeling of low molecular weight DNA from total testicular parenchyma isolated from the right heat/GnRH-agonist treated testis indicated increased low molecular weight DNA fragmentation occurred in the 185 and 370 base pair range (Figure 3). Fragmentation resembled Jurkat cell positive control DNA under serum starved conditions. A small amount of fragmentation (> 740 bp) was observed in the untreated testis, which was not observed in the Jurkat cell negative control DNA.

Figure 1: Brightfield photomicrographs depicting the eight stages of the cycle of the seminiferous epithelium in paraffin sections stained with PeriodicAcid-Schiff (PAS) and Toluidine blue. Wide view images were captured at 40X (Scalebar = 30 μm). Views of individual cells were captured at 100X (Scalebar = 10 μm).

Stage I: characterized by two generations of (L) leptotene and (P) pachytene spermatocytes. Sb1 round spermatids can be observed with a well-developed easily recognizable acrosomal cap. Developing tails are sometimes visible, although difficult to distinguish in paraffin sections.

Stage II: two generations of (L) leptotene and (P) pachytene spermatocytes. Sb2 spermatids become irregularly shaped as elongation and orientation begins. Acrosomal caps are plainly evident

Stage III: two generations of (Z) zygotene and (P) pachytene spermatocytes, and characteristic Sc spermatids which are oriented with acrosomal caps towards the basement membrane. No primary spermatocytes with meiotic figures are evident

Stage IV: contains the most germ cell types of any stage. B1 spermatogonia are evident (not shown) along the basement membrane next to zygotene primary spermatocytes. In the adluminal compartment, large pachytene spermatocytes begin their division and are seen with numerous meiotic figures (MF) (metaphase I and II), secondary spermatocytes (SS), newly formed Sa round spermatids, and Sc elongated spermatids

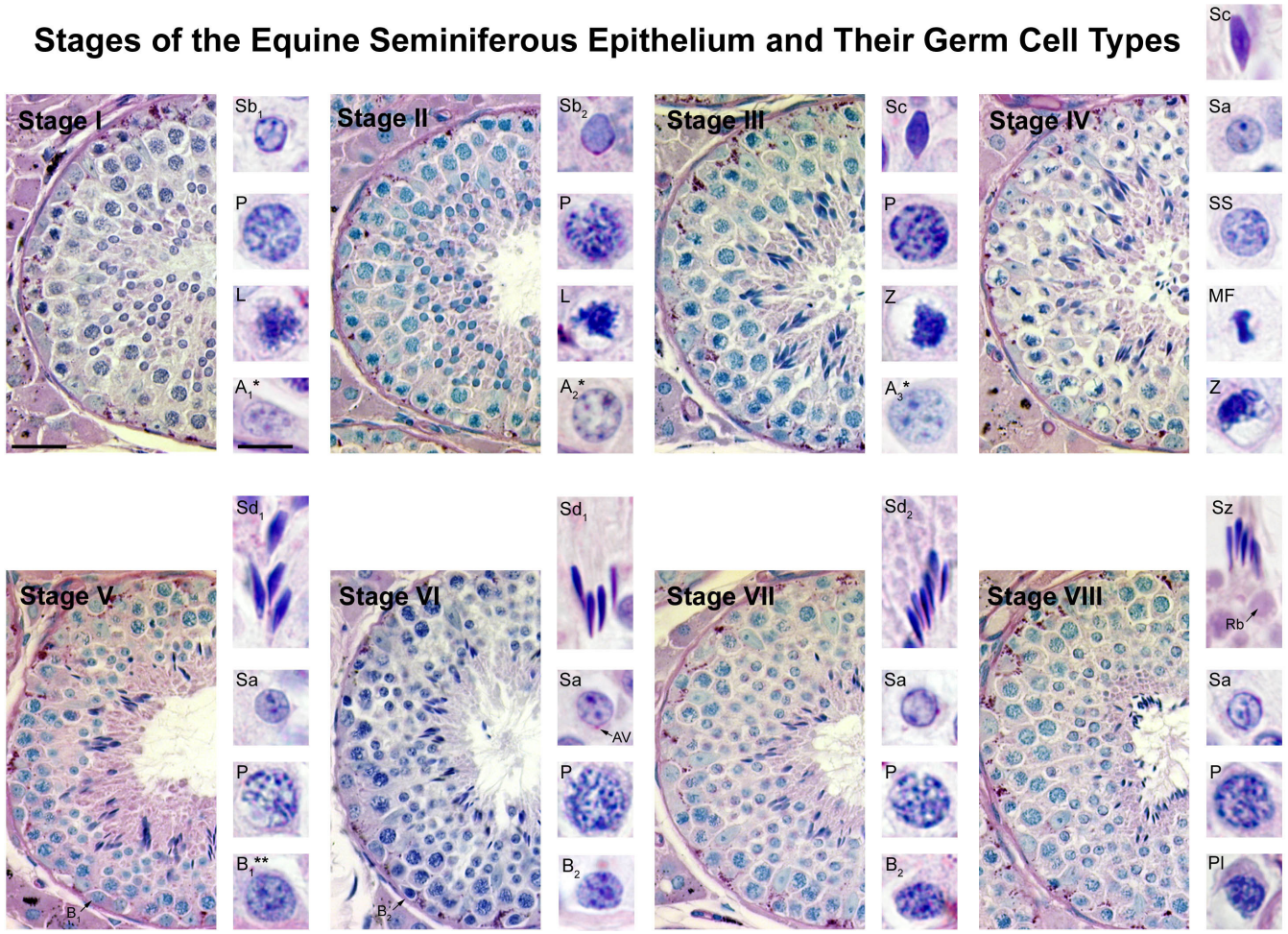
Stage V: B1 spermatogonia are still evident. Only one generation of pachytene spermatocytes may be found. Smooth nuclei of newly formed round spermatids have not begun formation of the acrosomal cap. Densely packed chromatin in Sd1 elongated spermatids may be found stacked in clusters, deeply embedded within the seminiferous epithelium.

Stage VI: Small, round B2 spermatogonia with small clumps of heterochromatin have formed and one generation of pachytene spermatocytes may be found near the basement membrane. Sa round spermatids can be observed with an acrosomic vesicle not yet touching the nucleus. Sd1 elongated spermatids begin their migration to the lumen.

Stage VII: B2 spermatogonia and one generation of pachytene spermatocytes may be found. Acrosomal caps of Sa round spermatids begin to flatten and cover the surface of the nucleus. Densely packed Sd2 elongated spermatids are migrating closer to the lumen

Stage VIII: B2 spermatogonia have divided to form the small round preleptotene (Pl) primary spermatocyte along the basement membrane. One generation of pachytene spermatocytes and late Sa round spermatids with developing caps can be found. Residual bodies (Rb) accumulate as mature spermatozoa are being released into the lumen as spermiation occurs.

Stages of the Equine Seminiferous Epithelium and Their Germ Cell Types



* A spermatogonia may be found in all eight stages (I-VIII)
** B_1 spermatogonia may be found in stages IV and V

4.3. Electron microscopy

Ultrastructural features of degenerating germ cells analyzed by transmission electron microscopy demonstrated morphological features that are consistent with apoptosis (Figure 4). Degenerating cells were observed with an intact, irregular plasma membrane and condensed chromatin localized near the nuclear margins. In all observed cases, degenerating cells were phagocytosed and contained within the Sertoli cell cytoplasm.

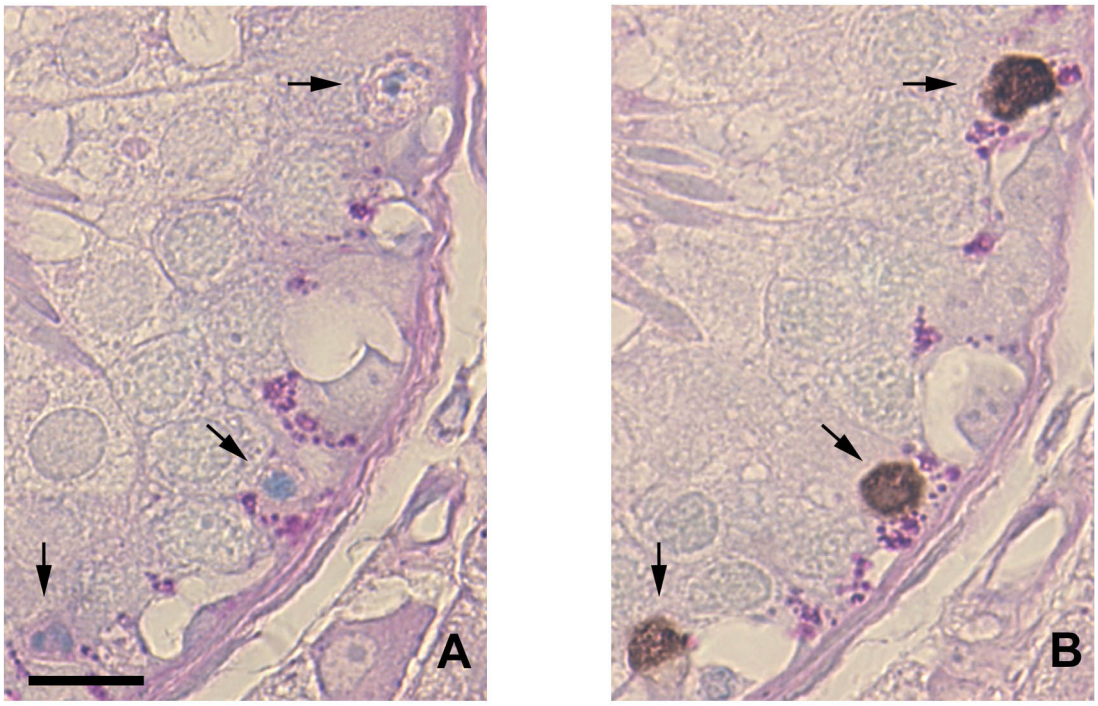


Figure 2: Pyknotic nuclei showing positive TUNEL staining in 3 µm serial sections. Panels A shows pyknotic nuclei (arrows) in a 3 µm paraffin section stained with PAS-Toluidine blue. Panel B shows the same nuclei as panel A in an adjacent serial section labeled with the TUNEL assay. Scale bar = 10µm.

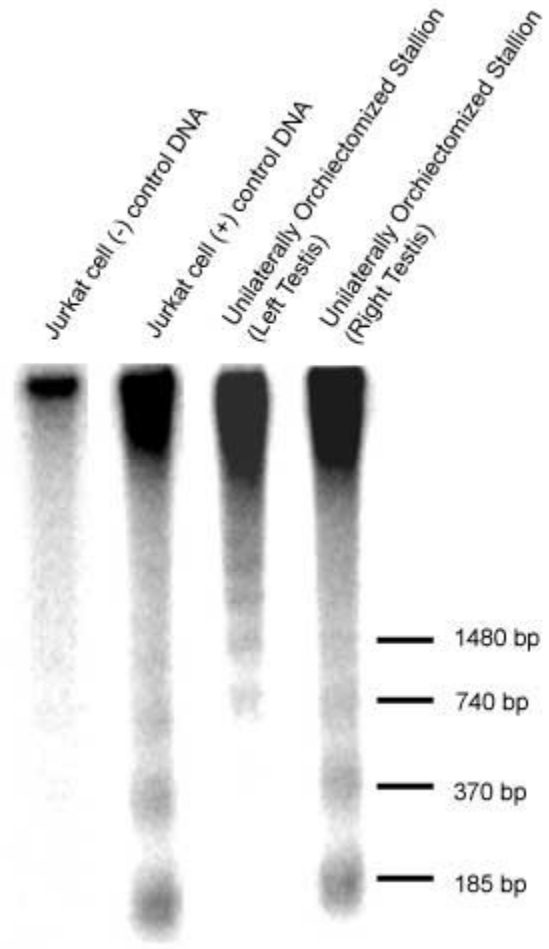


Figure 3: Isotopic end-labeling of low molecular weight DNA from total testicular parenchyma with or without heat/GnRH treatment indicated low molecular weight DNA fragmentation in multiples of 185 bp. DNA from the heat/GnRH treated stallion testis (right testis) is compared to the non-treated testis DNA (left testis) from the same stallion. An increase in DNA fragmentation occurred in the 185 and 370 bp size range. Heat/GnRH treated testis DNA was similar to Jurkat cell positive control DNA under serum starved conditions.

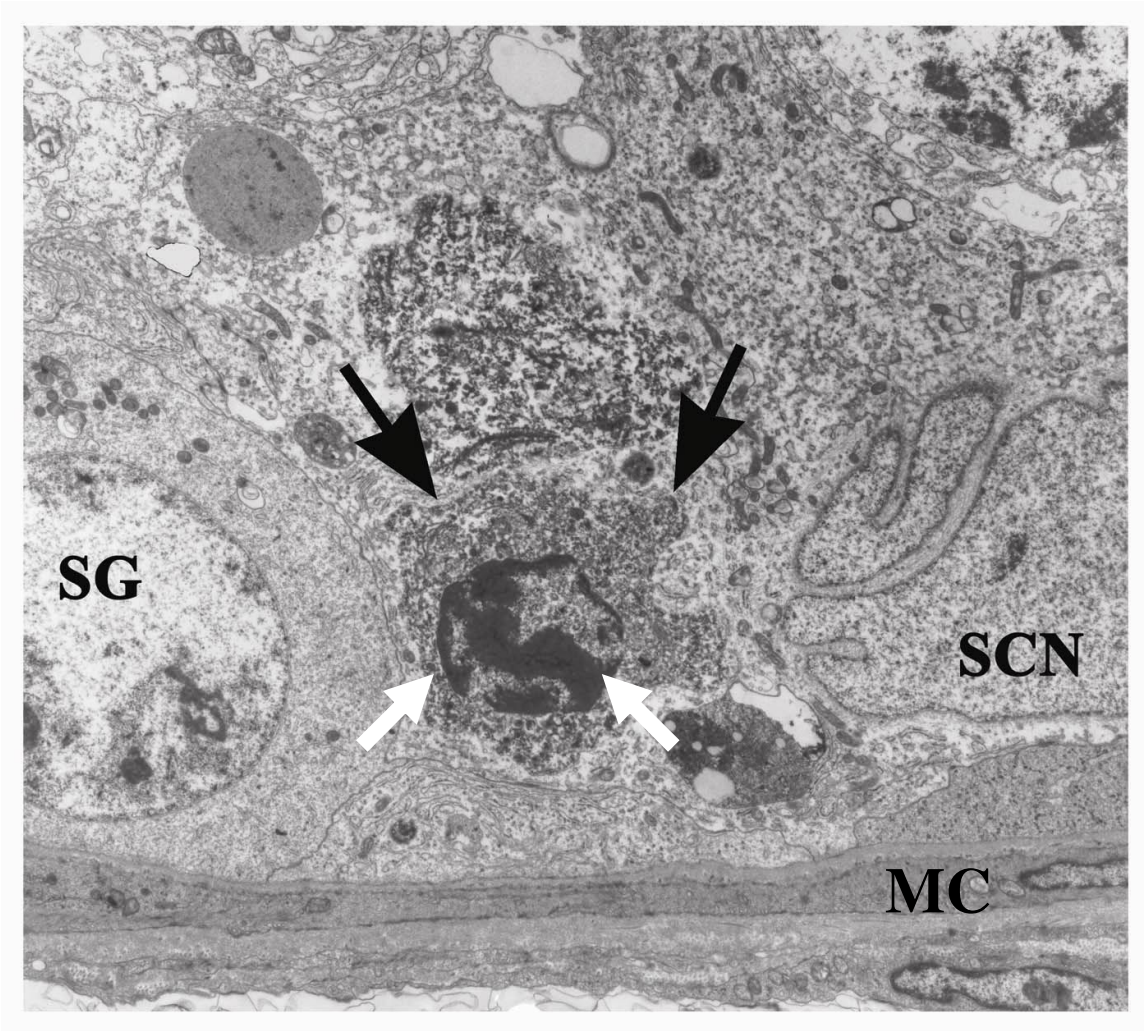


Figure 4: Transmission electron microscope image of the basal compartment of a seminiferous tubule from the testis of an adult stallion. Figure depicts a single apoptotic spermatogonia between a normal spermatogonial nucleus (SGN) and a normal Sertoli cell nucleus (SCN). A myoid cell (MC) marks the location in relation to the basal border of the seminiferous tubule. The apoptotic germ cell demonstrates an intact, irregular plasma membrane (dark arrows), and nuclear margination and condensation of chromatin (light arrows). The apoptotic germ cell has been completely phagocytized and is contained entirely within the Sertoli cell cytoplasm. Image captured at 8000X.

4.4. Quantification of germ cell apoptotic rates in reproductively normal stallions

No differences in the number of TUNEL-positive germ cells per 100 Sertoli cell nuclei were detected between right and left testes for the eight reproductively normal stallions ($P > 0.10$), so data from left and right testes of each horse were pooled to calculate overall means. Frequencies of seminiferous epithelial stages were similar to those reported by previous studies [54] (Figure 5). The mean number of Sertoli cell nuclei did not differ among seminiferous epithelial stages ($P=0.85$). Mean numbers of TUNEL-positive germ cells per 100 Sertoli cell nuclei were highest in stages IV (15.5 ± 1.0) and V (13.5 ± 1.1) of the seminiferous epithelial cycle ($P < 0.001$) (Figure 6). An intermediate level of apoptosis was detected in stage VI ($6.2 \pm .6$) ($P < .02$). Numbers of apoptotic germ cells in stages VII and VIII were not different from stages I to III ($P > .10$). The most common germ cells labeled by the TUNEL assay were spermatogonia and spermatocytes. A low frequency of round and elongated spermatids were observed to be labeled by the TUNEL assay.

5. Discussion

5.1. Confirmation of TUNEL labeling

Each of the analytical techniques employed in the current study confirmed that the TUNEL assay is an accurate method for quantifying apoptosis in germ cells of stallion testis. Apoptosis of germ cells was observed in both the left untreated and right treated testis of our 4-yr-old Thoroughbred stallion. However, the heat/GnRH-agonist treatment regime used in this study to generate high levels of apoptotic germ cells

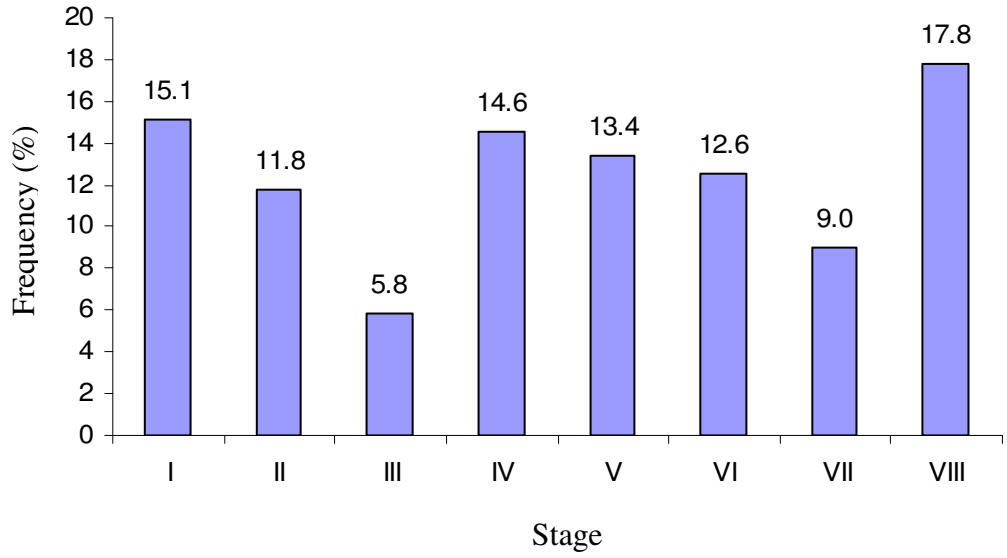


Figure 5: Observed histological frequencies of stages of seminiferous epithelium from round tubule cross-sections in eight reproductively normal stallions.

caused a three-fold increase in the number of TUNEL-positive germ cells per 100 Sertoli cell nuclei when compared to the untreated testis. Apoptotic germ cells in the treated testis were not randomly distributed throughout the seminiferous epithelium, but rather cohorts of 4 to 6 neighboring apoptotic germ cells within a given seminiferous tubule were often noted to be degenerating synchronously. This observation is consistent with a group of cells attached by intercellular bridges synchronously undergoing apoptosis [100]. The high level of apoptosis unfortunately precluded identification of the stage of seminiferous epithelium most affected by the treatment due to missing generations of germ cells and tubule disorganization. Our findings support previous studies that used heat or pituitary suppression as an effective means for inducing testicular germ cell

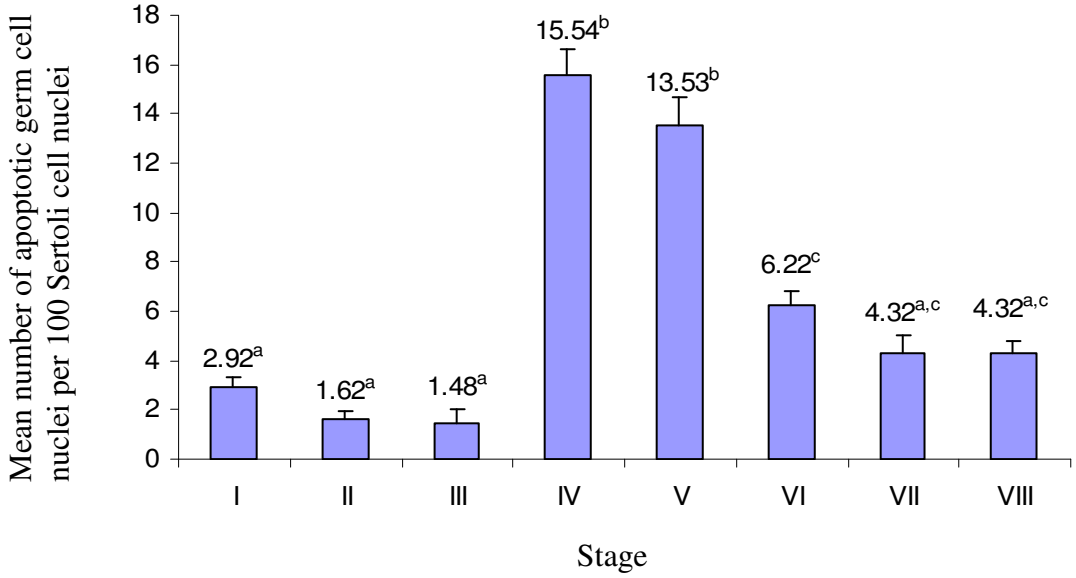


Figure 6: Mean number of apoptotic testicular germ cells per 100 Sertoli cell nuclei according to stage of seminiferous epithelium. Mean numbers of TUNEL-positive germ cells per 100 Sertoli cell nuclei were highest in stages IV (15.54 ± 1.04) and V (13.53 ± 1.11) of the seminiferous epithelial cycle ($P < .001$). An intermediate level of apoptosis was detected in stage VI ($P < .02$). Numbers of apoptotic germ cells in stages VII and VIII were not different from stages I-III ($P > .10$). † Means with different superscripts are different [a,b,c] ($P<.05$).

apoptosis in mature animals [71,92-94].

In the histological comparison of TUNEL-positive cells with the same cells in unstained serial sections, 97% of TUNEL-positive germ cells appeared pyknotic in adjacent, unlabeled serial sections. In rare instances, pyknotic nuclei were not labeled by the TUNEL assay. This might be explained by previous studies which have shown DNA fragmentation to be a relatively late event during the time-course of apoptotic

processes [101]. It likely that these unlabeled pyknotic cells had commenced apoptotic processes, but had yet to activate endogenous nuclear DNA fragmentation mechanisms.

Low molecular weight DNA analysis is a reputable method for establishing non-random patterns of DNA fragmentation. Other biological processes (i.e., necrosis) would theoretically yield tremendous amounts of random DNA fragmentation and generate a non-uniform DNA smear during gel electrophoresis rather than the distinct ladder pattern commonly observed. However, ladder assays for detection of apoptosis have limited capabilities. In the testis, DNA is generally extracted from whole tissue lysates or, at best, isolated segments of seminiferous tubules staged by trans-illumination microscopy. This negates the ability to ascertain the exact cell type(s) undergoing apoptosis using this particular methodology. In our study, an observable difference in DNA fragmentation occurred in the testis from the heat/GnRH treated stallion (Figure 3). A minor amount of DNA fragmentation (> 750 base pairs) was detected in the non-treated testis and should be expected due to the common occurrence of apoptosis in normal stallion testes.

Our findings indicate that apoptotic germ cells from stallion testis had increased electron density in nuclear and cytoplasmic regions, as well as other ultrastructural features consistent with those described by others [102](Figure 4). While the electron microscope is a precise method for identification of apoptotic cells, it is not suited for quantitative analysis of large numbers of apoptotic cells [103].

5.2. Germ cell apoptotic rates in reproductively normal stallions

Our results support the work of previous studies, in which apoptosis of germ cells coincides with mitotic and meiotic peaks [49]. In normal stallion testis, apoptotic germ cells were observed at the highest frequency in stage IV. A significant proportion of the labeled cells could be clearly identified as late pachytene, diplotene, and metaphase spermatocytes (Figure 7A). In stage V, apoptotic cells were observed most frequently and identified as spermatogonia. Since a previous study in the stallion has shown stage V and VI to contain high numbers of B1 and B2 spermatogonia [104], and that during the breeding season, an increase in A spermatogonia is coupled to an increase in degeneration of B2 spermatogonia [40,104], apoptotic cells in the basal compartment of stages V and VI are presumably B1 and B2 spermatogonia (Figure 7B), although other earlier spermatogonia cannot be excluded. A small number of large apoptotic pachytene spermatocytes from the previous stage IV were observed early in stage V and contributed to the elevated level of apoptosis in this stage, which has been similarly suggested for the rat [49]. The stages of the seminiferous epithelium containing early prophase spermatocytes (preleptotene, leptotene, and zygotene spermatocytes; stages I to III and VII to VIII) tended to have lower rates of apoptosis as has been described in other species [49,50,105,106].

Varying rates of apoptosis in different germ cell types suggest that critical mitotic and meiotic checkpoints probably exist [39,69]. For the stallion, it appears that regulation of spermatogonial proliferation takes place in part during the differentiation of B spermatogonia; however, the extent of apoptosis in earlier forms of spermatogonia

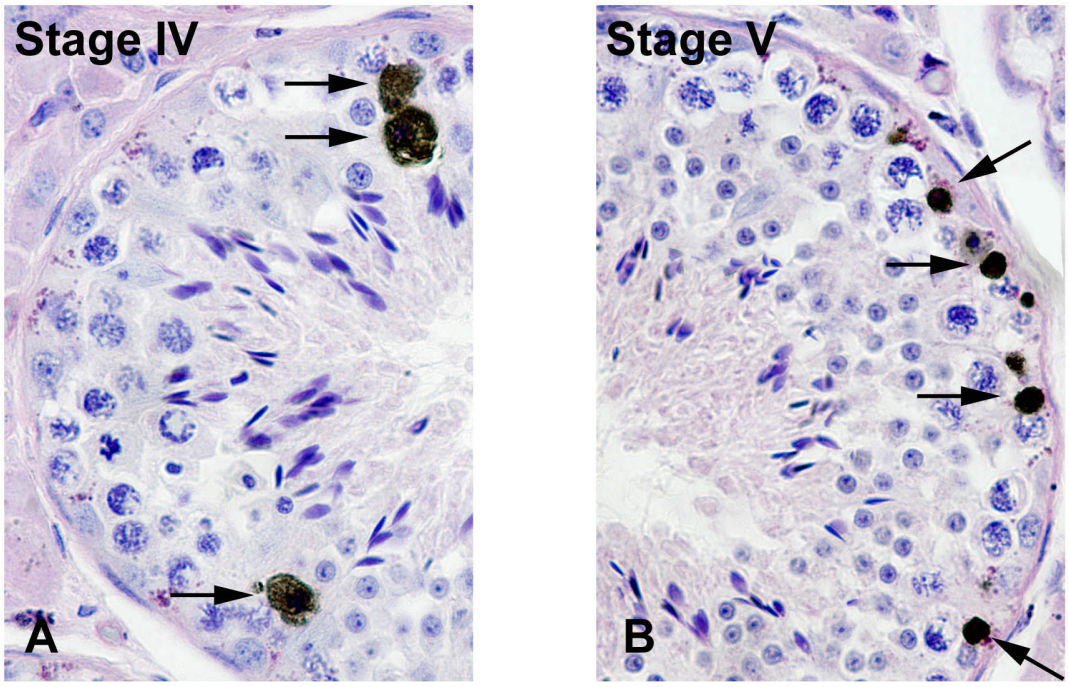


Figure 7: Panel A shows a stage IV tubule with a full complement of germ cell types. Large apoptotic pachytene spermatocytes can be clearly identified and are the most common cell type labeled with the TUNEL assay in this stage. Panel B shows a stage V tubule with spermatogonia (presumably B1) being the most commonly labeled germ cell type. Residual apoptotic pachytene spermatocytes from the previous stage were also observed early in stage V. Scale bar = 20µm.

must be considered since even slight rises in the level of apoptosis in these cell types could have dramatic effects on overall germ cell numbers. During meiosis in the stallion, regulation by apoptosis appears to occur most frequently after DNA synthesis (S-phase) and during the transition from G2 to M phase of the cell cycle.

Apoptosis of germ cells during specific stages of the cell cycle may represent removal of germ cells with incomplete genetic components or chromosomal abnormalities [101,107]. Other possible roles for apoptosis in the testis include the

seasonal modulation of spermatogenesis [51-53], a regulatory mechanism to maintain optimal germ cell numbers per Sertoli cell [5,108,109], and/or to maintain architecture and position of germ cells in the seminiferous epithelium [110].

A very low rate of TUNEL-positive round and elongated spermatids was observed. One explanation may be that the TUNEL assay in our experiment was not able to adequately label these cells due to either the compact nature of the DNA in spermatids and/or the limited mRNA production in spermatids which is generally thought to be necessary for apoptotic death [111]. Alternatively, perhaps the apoptotic rate of spermatids may have been very low in this group of reproductively normal stallions. Previous stereological studies in our lab [40,85] noted that high sperm-producing stallions did not suffer germ cell losses during spermiogenesis, while low sperm-producing stallions suffered significant germ cell losses during spermiogenesis. Further research remains to be performed to evaluate the role of apoptosis in loss of round and elongated spermatids for the stallion, particularly in stallions producing low numbers of normal sperm.

In conclusion, germ cell degeneration in the stallion takes the form of apoptosis and its classical morphological characteristics. The use of the TUNEL assay to quantify levels of germ cell death in the testis is a valuable tool to quantitate levels of apoptosis in stallion testicular germ cells. The TUNEL assay is a repeatable and reliable method for labeling the later stages of germ cell death after DNA fragmentation has occurred. Cells with normal morphological appearance were never labeled by the TUNEL assay. In stallions with normal semen quality and testis size, the presence of TUNEL-positive

germ cells implies an inherent nature of apoptosis during normal spermatogenesis. Apoptosis during spermatogenesis of the normal stallion occurs most frequently during stages IV, V, and to a lesser extent, VI. Quantitative levels of apoptosis in these reproductively normal stallions may now be used in future studies for comparison to clinically identified cases of idiopathic subfertility. Establishing basal levels of germ cell apoptosis is a critical step towards understanding the seminiferous tubule biology and role of apoptosis in regulating germ cell numbers during spermatogenesis in the stallion.

CHAPTER IV

RELATIONSHIP OF GERM CELL APOPTOSIS TO SPERMATOGENIC EFFICIENCY IN STALLIONS WITH REDUCED SEMEN QUALITY

1. Overview

To examine the relationships between germ cell apoptotic rate and spermatogenic efficiency, seminal traits were assessed to classify stallions into normal (n = 10) or reduced (n = 7) quality (low number of normal, motile sperm) semen groups. Circulating concentrations of selected pituitary- and gonadal-derived hormones were determined. Stallions were castrated, and testes were processed for quantification of apoptotic germ cells (number of TUNEL-positive germ cells/100 Sertoli cell nuclei) and histomorphometric assessment of sperm production rates. Apoptotic rates were higher ($P < 0.05$) for stages IV-VI and stage VIII seminiferous tubules in stallions with reduced semen quality. Daily sperm production (DSP) per gram and per testis were lower ($P < 0.05$) for stallions with reduced semen quality. Both groups of stallions suffered declines in spermatogenic potential during spermatocytogenesis and spermiogenesis, but the rate of decline did not differ between groups. Among all stallions, regression analyses revealed negative linear relationships ($P < 0.05$) for germ cell apoptotic rate with DSP/g ($r^2 = 0.54$), DSP/testis ($r^2 = 0.77$), daily sperm output ($r^2 = 0.39$), number of progressively motile sperm ($r^2 = 0.53$) and number of morphologically normal sperm ($r^2 = 0.68$) in ejaculates. Mean circulating concentrations of inhibin were lower ($P < 0.001$) for stallions ejaculating reduced quality semen. Apoptotic rate was negatively correlated with concentrations of inhibin ($P < 0.02$) and estradiol-17β ($P < 0.01$), and positively

correlated with concentrations of LH ($P < 0.04$) and FSH ($P < 0.02$). Elevated rates of germ cell apoptosis explain, in part, the low spermatogenic efficiency associated with ejaculation of reduced quality semen in stallions.

2. Introduction

Apoptosis (programmed cell death) is a well-defined, highly complex physiological process that serves to eliminate damaged, diseased, or superfluous cells from various tissues of the body (for a review see [112]). Apoptosis in testicular germ cells has been demonstrated in many mammalian species [43-48], including the horse [113]. Spermatogenesis is a relatively inefficient process, resulting in the estimated loss of 25-75% of the potential number of mature sperm produced in the adult testis [38,39]. The failure to reach potential sperm production has been shown to result from an overproduction of early germ cell types beyond that which can be sustained by supporting Sertoli cells [40] and a varied efficiency of its mitotic and meiotic capabilities that can partly overcome early losses [105]. Apoptosis has been implicated as the primary mechanism for removal of developing germ cells from the seminiferous epithelium and has also been shown to be necessary for the development of normal spermatogenesis [5,41,42].

The stage of seminiferous epithelium reflects multiple germ cell progeny at different developmental steps of differentiation. During normal spermatogenesis, elevated levels of apoptosis generally correspond to stages in which mitotic and meiotic peaks of germ cell development occur [49]. This suggests that some germ cell types are

more susceptible to death at specific steps of differentiation. Alteration in apoptotic germ cell death has been identified as a factor in cases of clinically identified male infertility/subfertility and has been linked either directly or indirectly to various forms of testicular pathology such as testicular degeneration [114-116], germ cell tumors [55], cryptorchidism [56], torsion of the spermatic cord [57,58], and hypospermatogenesis [59,60]. Men with low fertility experience altered levels of apoptosis (either the absence of appropriate apoptosis or its inappropriate occurrence [6]) that may be initiated by signals from Sertoli cells, neighboring germ cells, hormonal signaling, or environmental factors [117].

Increased germ cell losses (described as germ cell degeneration) during spermatogenesis in stallions has been associated with low sperm production [85], and reduced semen quality [81]. We hypothesize that increased germ cell apoptotic rates explain low spermatogenic efficiency in stallions with reduced semen quality. The objectives of this study were to: 1) compare germ cell apoptotic rates within each stage of spermatogenesis in stallions ejaculating normal or reduced quality semen, and 2) quantify the relationship of germ cell apoptotic rate with sperm production, semen quality, and circulating reproductive hormones in the stallion.

3. Materials and Methods

3.1. Animals and reproductive characterization

Seventeen light breed stallions (Quarter Horse, Arabian, Thoroughbred or crossbred) aged 2-20 years and maintained in southeast Texas were used in this study.

Stallions were fed coastal Bermuda grass hay and grain supplement sufficient to maintain good body condition. Some data from eight stallions with normal semen quality were reported in a previous study by our lab [113].

To characterize semen quality, semen was collected daily for 10 days to stabilize extragonadal sperm reserves and establish daily sperm output (DSO) as a measure of sperm production [118]. Semen was collected using an ovariectomized mount mare and a Missouri-model artificial vagina (Nasco, Ft. Atkinson, WI, USA) equipped with an in-line nylon micromesh filter (Animal Reproduction Systems, Chino, CA, USA). Volume of gel-free semen was measured in a graduated cylinder, and sperm concentration in the gel-free ejaculate was measured photometrically (Densimeter, Model 534B, Animal Reproduction Systems, Chino, CA, USA). Total sperm number was calculated as the product of sperm concentration and volume of gel-free semen. An aliquot of gel-free semen was extended to 25 million sperm per ml in nonfat dried milk solids, glucose semen extender (EZ Mixin-CST, Animal Reproduction Systems, Chino, CA, USA). The percentage of sperm that were progressively motile was determined using a computerized sperm motility analyzer (HTM IVOS; Version 10.8; Hamilton Thorne Research, Beverly, MA, USA). The number of progressively motile sperm in each ejaculate was calculated as the product of total sperm number in the ejaculate and the percentage of progressively motile sperm. An additional aliquot of gel-free semen was diluted in 2% buffered formal-saline solution for classification of morphologic features of 100 sperm using a differential interference contrast microscopy at 1250 X magnification. The number of morphologically normal sperm in each ejaculate was

calculated as the product of total sperm number and the percentage of morphologically normal sperm.

At DSO, stallions ejaculating normal quality semen (> 60% progressively motile sperm and > 55% morphologically normal sperm) comprised the normal group (n=10), while stallions ejaculating reduced quality semen (≤ 55% progressive sperm motility and ≤ 39% morphologically normal sperm) comprised the group with reduced semen quality (n=7). Fertility data were not available for stallions in this trial.

3.2. Blood and testis samples

Prior to castration, heparinized blood samples were collected by jugular venipuncture at hourly intervals between 0900 and 1200 hr. Equal volumes of plasma were pooled from each of four samples and frozen (-80^{o}C) until assayed by radioimmunoassay for LH [119], FSH [119], testosterone [120], estradiol-17β [121] and inhibin [122] concentrations. Blood samples were not available for three stallions with reduced semen quality (as described below) and for one stallion with normal semen quality.

Testes were obtained by surgical castration in lateral recumbency under general anesthesia (1.0 mg/kg xylazine iv; 2.0 mg/kg ketamine iv). Both testes were removed from 14 stallions, while a single testis was obtained from three stallions with reduced semen quality (one descended testis from a unilateral cryptorchid; and a single testis from two stallions based on clinical availability). Each testis was weighed and sections (2 cm x 2 cm x 3 mm) of testicular parenchyma were obtained from the mid-testis region

for immersion fixation for 24 hours in 4% (v/v) paraformaldehyde or 2% (v/v) glutaraldehyde (Sigma-Aldrich Co., St. Louis, MO, USA) in cacodylate buffer. Each testis was then stripped of the visceral tunic and the tunic was weighed. Parenchymal weight for each testis was calculated by subtraction of tunic weight from testis weight. Each stallion had sperm in their ejaculates, and a full complement of germ cells within seminiferous tubule cross-sections which excluded obstructive azoospermia and Sertoli cell only syndrome as causes for the reduced semen quality.

3.3. Histology and the detection of apoptosis

Immunohistochemical detection of apoptosis using the TUNEL assay (Tdt-mediated dUTP nick end labeling) for the detection of fragmented DNA was performed according to Heninger et al. [113] using an Apoptag® TUNEL assay (Intergenco, Purchase, NY, USA). To quantify germ cell apoptotic rates, seminiferous tubules were evaluated using brightfield-illumination under oil immersion at 630 X magnification (Axioplan2 Photomicroscope, Zeiss Inc., New York, NY). One hundred round cross-sections of seminiferous tubules from each testis were classified by stage of the seminiferous epithelium (I-VIII) according to Swierstra et al [22] and Johnson et al [21]. A Periodic Acid –Schiff (PAS) staining technique proposed by Clermont and Leblond [32] was used for more critical assessment of acrosomal morphology. Only round tubule cross-sections containing at least 75% of a respective stage were evaluated. The apoptotic rate for each stage was expressed as the number of TUNEL-positive germ cells per 100 Sertoli cell nuclei to compensate for histological shrinkage and differences in

tubule diameter. Stage of seminiferous epithelium, position of germ cells in the seminiferous epithelium and nuclear diameter were used as criteria for identification of germ cell types undergoing apoptosis.

3.4. Stereology and enumeration of germ cell and Sertoli cell numbers

Three post-glutaraldehyde-fixed parenchymal samples (10 mm x 5 mm x 2 mm) from each testis were further fixed in osmium tetroxide and embedded in Epon 812. The total number of Sertoli cells, type B spermatogonia (B1, B2) early (preleptotene and leptotene plus zygotene) primary pachytene spermatocytes, late (pachytene plus diplotene) primary spermatocytes, and spermatids with spherical nuclei were enumerated by stereology of Epon sections. Using a previously established point-counting method [123], nuclear volume density (% parenchyma occupied by each respective germ cell nuclei type) was determined using a 50-point grid (5000 points per testis) on 0.5-μm toluidine blue-stained sections observed in brightfield at 1000 X magnification. The average nuclear volume for each cell type was determined using the formula for a sphere ($4/3\pi r^3$) after obtaining mean maximum diameters of respective germ cell types in 20-μm unstained Epon sections [40,124]. Measurements of maximum nuclear diameter were accomplished at a magnification of 1000 X using a microscope fitted with a computerized digital imaging system fitted with Nomarski optics. The number of a particular germ cell type was determined by dividing the product of nuclear volume density, parenchymal volume (1.05 ml/g), nuclear diameter, and a histological correction factor for section thickness (0.91) by the average volume of a single nucleus for that

respective cell type. The daily sperm production potential (per gram and per testis) during spermatogenesis was estimated after correction of each cell type for its expected lifespan (B1 spermatogonia = 2.8d, B2 spermatogonia = 3.2d, early (preleptotene, leptotene and zygotene) primary spermatocytes = 8.1d, late (pachytene) primary spermatocytes = 12.2d, round spermatids = 8.1d) [54,125] and theoretical yield (B1 = 16, B2 = 8, Early = 4, Late = 4, Round = 1). The decline in potential DSP during spermatogenesis is defined by the loss (degeneration) of potential germ cells based on the theoretical maximum number of B1 spermatogonia.

3.5. Determination of DSP by counting of maturation phase spermatids

Estimation of daily sperm production (DSP) was performed by enumeration of maturation-phase (elongated, homogenization-resistant) spermatids according to previously established methods [126]. Briefly, 0.2-0.5 grams of 2% glutaraldehyde-fixed testicular parenchyma were homogenized in a Waring blender for 6 minutes in 100 ml of fluid containing 150 mM NaCl, 0.05% (v/v) Triton X-100 and 3.8 mM NaN_3. Maturation-phase elongated spermatids characteristic of stages III-VIII were enumerated using duplicate evaluations (at least 4 cytometer chambers per evaluation). Daily sperm production per gram of parenchyma (DSP/g) was calculated for each horse as the number of elongated, homogenization-resistant spermatids divided by the product of the weight of the homogenized tissue and the lifespan of the spermatids (8.2 days). Daily sperm production per testis was calculated as the product of DSP/g and parenchymal weight.

3.6. Statistics

Data for the number of apoptotic germ cells/100 Sertoli cell nuclei by stage of seminiferous epithelium and potential DSP during spermatogenesis were analyzed using general linear models procedures (SAS® Statistical Software; Cary, NC, USA, 2000) in a split-plot design using semen quality (SQ: normal vs. reduced), testis nested within SQ, stage and SQ*stage in the statistical model. Testis nested within SQ was used as the error term to detect differences in SQ. All other terms were tested using the residual error term.

To detect differences between groups (normal vs. reduced semen quality), data for the number of apoptotic germ cells/100 Sertoli cell nuclei, potential sperm production (DSP/g, DSP/testis), seminal parameters [daily sperm output, percentage (transformed to arcsin) and number of progressively motile sperm, percentage (transformed to arcsin) and number of morphologically normal sperm] and plasma hormone concentrations (testosterone, estradiol 17β, LH, FSH, and inhibin) were analyzed by an independent samples t-test (SPSS© Inc.; Chicago, Illinois, USA, 2001). The Levene's test was used to test for equality of variances. Means were considered significantly different at $P < 0.05$. Linear regression analysis revealed similar trends between groups of stallions, so data were pooled to evaluate relationships for the mean level of germ cell apoptosis (across all stages) with total number of sperm per ejaculate, number of progressively motile sperm per ejaculate, number of morphologically normal sperm per ejaculate, DSP/g, and DSP/testis. Correlations for the mean level of germ cell apoptosis with circulating hormone concentrations were examined.

4. Results

Mean values for age, testis, semen and endocrine parameters for stallions ejaculating semen of normal or reduced quality are presented in Table 1. Ranges for parenchymal weight, apoptotic rates of germ cells, DSP and DSO by stallion group are presented in Table 2. Stallions ejaculating semen of reduced quality had smaller testes ($P < 0.001$), produced fewer sperm per gm ($P < 0.001$) and per testis ($P < 0.001$), and ejaculated fewer total sperm ($P < 0.03$), fewer progressively motile sperm ($P < 0.001$) and fewer morphologically normal sperm ($P < 0.001$) than stallions ejaculating semen of normal quality.

Mean number of apoptotic germ cells/100 Sertoli cell nuclei in stallions with normal and reduced semen quality is depicted in Figure 8. Greater apoptotic rates were present in stages IV-VI and stage VIII tubules for stallions with reduced semen quality as compared to stallions with normal semen quality ($P < 0.05$). No differences in apoptotic rate existed between stallions with normal and reduced semen quality for Stages I-III or Stage VII tubules ($P > 0.10$).

Table 1. Mean (± SEM) values for age, testis parameters, semen parameters, and endocrine status, in stallions with normal or reduced semen quality.

	Normal Semen Quality	Reduced Semen Quality	Significance
Number of stallions	10	7	
Age (yr)	6.8 ± 1.4	13.2 ± 2.1	P < 0.001
Testis parameters			
Parenchymal Weight (g)	203.5 ± 7.8	132.5 ± 16.8	P < 0.001
Daily Sperm Production (10^6)[a]			
Per gram	15.5 ± 0.6	10.9 ± 1.4	P < 0.001
Per testis	3199 ± 158	1615 ± 292	P < 0.001
Semen Parameters			
Daily Sperm Output (10^6)	6081 ± 504	4029 ± 928	P < 0.03
Progressively Motile Sperm (%)	80 ± 3	35 ± 9	P < 0.001[b,c]
Progressively Motile Sperm (10^6)	4357 ± 432	1648 ± 544	P < 0.001
Morphologically Normal Sperm (%)	75 ± 3	25 ± 5	P < 0.001[c]
Morphologically Normal Sperm (10^6)	4137 ± 380	1118 ± 270	P < 0.001[b]
Endocrine Status			
Number of Stallions	9	4	
Testosterone (ng/ml)	1.02 ± 0.11	1.63 ± 0.71	NS
Estradiol 17β (pg/ml)	48 ± 4	35 ± 11	P = 0.10[b]
LH (ng/ml)	6.77 ± 1.69	8.70 ± 1.84	NS
FSH (ng/ml)	4.91 ± 1.62	11.55 ± 5.67	P = 0.08
Inhibin (ng/ml)	3.48 ± 0.55	1.56 ± 0.41	P < 0.001

[a] Determined by counting of elongated spermatids in testicular homogenates
[b] P value reported assuming unequal variance (all others assume equal variances)
[c] Transformed to arcsin for evaluation

Table 2. Ranges in parenchymal weight, apoptotic rates of germ cells, daily sperm production (DSP)[a] and daily sperm output (DSO) for stallions with normal or reduced semen quality.

	Normal Semen Quality (n=10)	Reduced Semen Quality (n=7) **	# of stallions ***
Total parenchymal weight (g)*	311 – 517	123 – 390	2
Apoptotic germ cells/100 Sertoli cell nuclei	4.1 - 11.6	5.5 – 18.1	1
DSP/horse (10^6)[a]	4552 – 7918	594 – 5768	2
DSP/g (10^6)[a]	13 – 18.1	4.8 – 14.8	2
Total DSO (10^6)	3612-8534	0.0008 – 7198	4

[a] Based on the number of elongated spermatids in testicular homogenates

* Three stallions contributing a single testis were excluded from the range

** Number of stallions with reduced semen quality with values in the range observed for stallions with normal semen quality

*** One stallion with reduced semen quality (low number of normal, motile sperm) had values in the normal range for each parameter

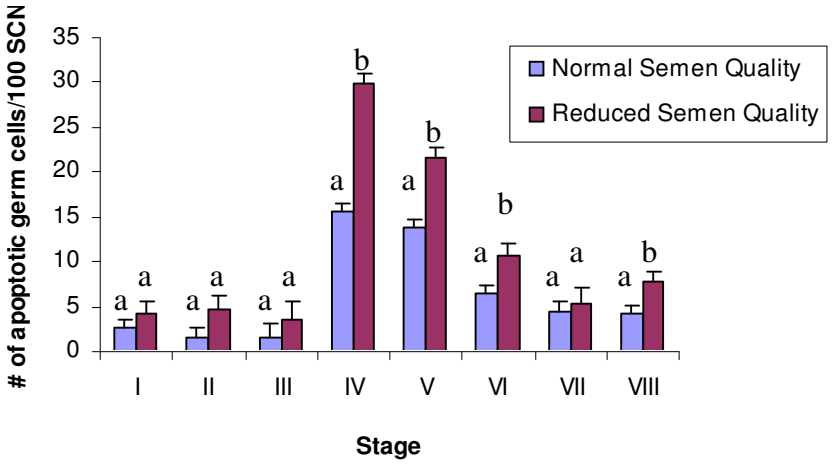


Figure 8: Mean number of TUNEL-positive (apoptotic) germ cells per 100 Sertoli cell nuclei (SCN) by stage (I-VIII) in stallions with normal or reduced semen quality. [a,b] Within stage, means with different superscripts are different ($P < 0.05$).

Mean potential daily sperm production per gram (DSP/g) and per testis (DSP/testis) for various germ cell types during spermatogenesis in stallions with normal and reduced semen quality are illustrated in Figures 9 and 10. Potential DSP/g and DSP/testis were decreased ($P < 0.001$ and $P < 0.001$, respectively) during spermatogenesis for stallions ejaculating reduced quality semen compared to stallions ejaculating normal quality semen. Both groups of stallions exibited declines in potential DSP/g and DSP/testis during spermatocytogenesis (division of B1 spermatogonia into B2 spermatogonia) (42% and 54% decline for stallions with normal or reduced semen quality, respectively), and during spermiogenesis (round to elongated spermatids) (34% and 33% decline for stallions with normal or reduced semen quality, respectively), but

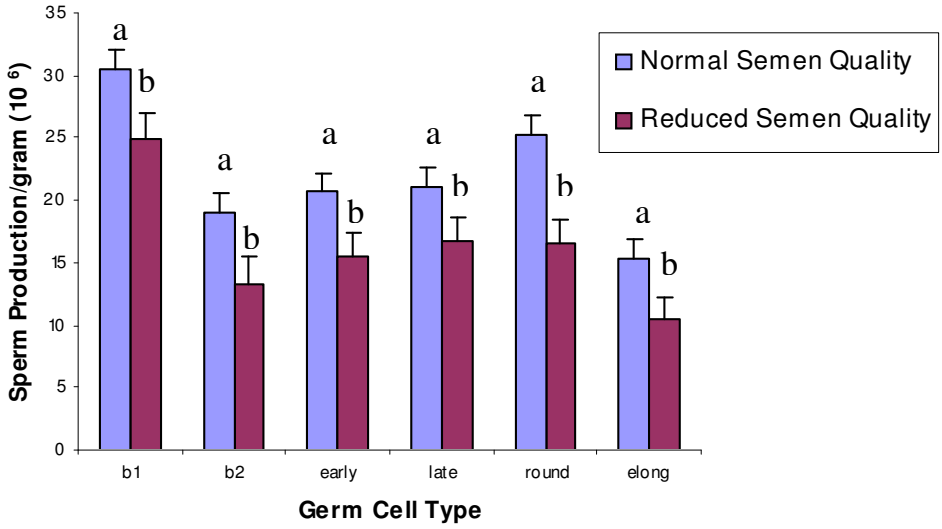


Figure 9: Mean potential daily sperm production per gm (DSP/gm) of testis by germ cell type during spermatogenesis in stallions with normal and reduced semen quality. b1 = B1 spermatogonia; b2 = B2 spermatogonia; early = early spermatocytes; late = late spermatocytes; round = round spermatids; elong = elongated spermatids. [a,b] Within germ cell type, means with different superscripts differ ($P < 0.05$).

rates of decline did not differ between groups ($P = 0.15$; Figure 11). Neither group of stallions experienced declines in potential DSP/g or DSP/testis during meiosis.

Mean circulating concentrations of inhibin were lower ($P < 0.001$) in stallions ejaculating semen of reduced quality when compared to those ejaculating semen of normal quality (Table 1). There was a tendency for mean circulating estradiol concentrations to be lower ($P = 0.10$), and for mean circulating FSH concentrations to be higher ($P = 0.08$) in stallions ejaculating reduced quality semen (Table 1). No differences were detected in mean circulating concentrations of testosterone or LH ($P > 0.10$) between stallions ejaculating reduced quality of normal semen.

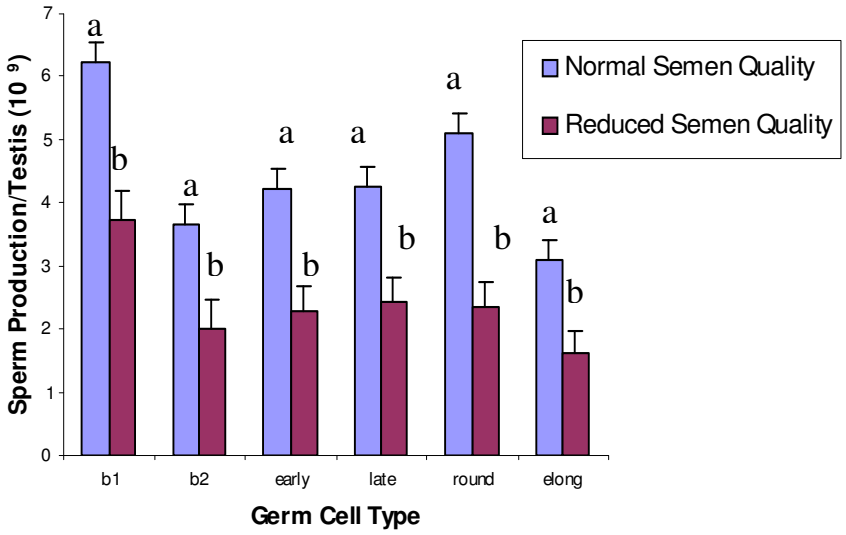


Figure 10: Potential daily sperm production per testis (DSP/testis) by germ cell type during spermatogenesis in stallions with normal and reduced semen quality. b1 = B1 spermatogonia; b2 = B2 spermatogonia; early = early spermatocytes; late = late spermatocytes; round = round spermatids; elong = elongated spermatids. [a,b] Within germ cell types, means with different superscripts differ ($P < 0.05$).

Among all stallions, linear regression analyses revealed negative relationships for germ cell apoptotic rate (number of apoptotic germ cells/100 Sertoli cell nuclei) with DSP/g ($r^2 = 0.54$; $P < 0.05$; Figure 12A), DSP/testis ($r^2 = 0.77$; $P < 0.05$; Figure 12B), DSO ($r^2 = 0.39$; $P < 0.05$; Figure 12C), number of progressively motile sperm in ejaculates at DSO ($r^2 = 0.53$; $P < 0.05$; Figure 12D), and number of morphologically normal sperm in ejaculates at DSO ($r^2 = 0.68$; $P < 0.05$; Figure 12E). Negative correlations were also detected for germ cell apoptotic rate with circulating concentrations of estradiol-17β ($r^2 = 0.48$; $P < 0.01$) and inhibin ($r^2 = 0.40$; $P < 0.02$). Conversely, positive correlations were detected for germ cell apoptotic rate with circulating concentrations of LH ($r^2 = 0.29$; $P < 0.04$) and FSH ($r^2 = 0.38$; $P < 0.02$).

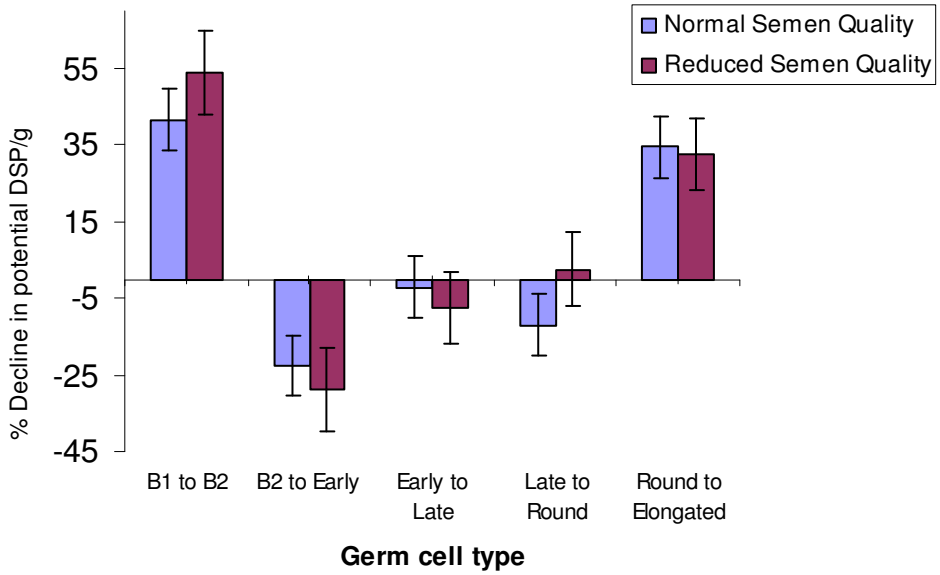
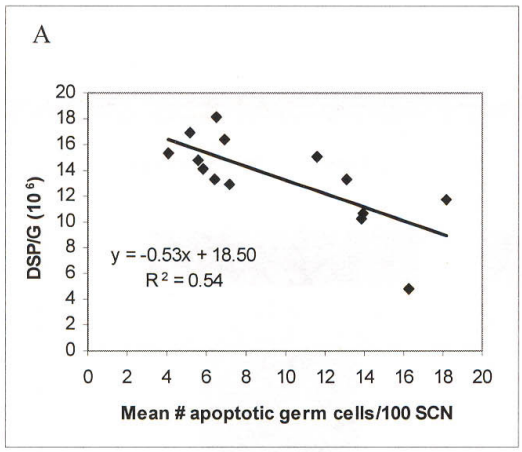


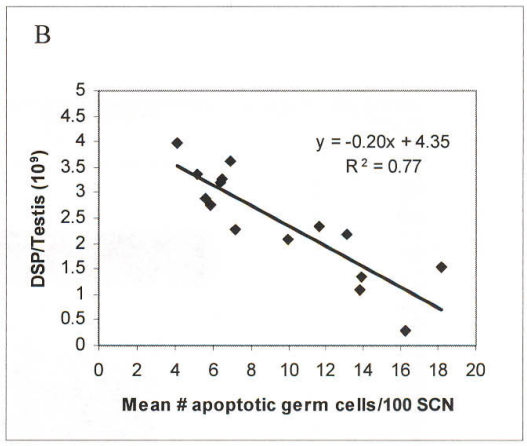
Figure 11: Mean rates of decline in potential daily sperm production based on the number of b1 spermatogonia during spermatogenesis in stallions with normal or reduced semen quality. B = type B spermatogonia; Early = early primary spermatocytes; Late = late primary spermatocytes; Round = round spermatids; Elongated = elongated spermatids. Means did not differ ($P < 0.05$) between semen quality groups within germ cell type.

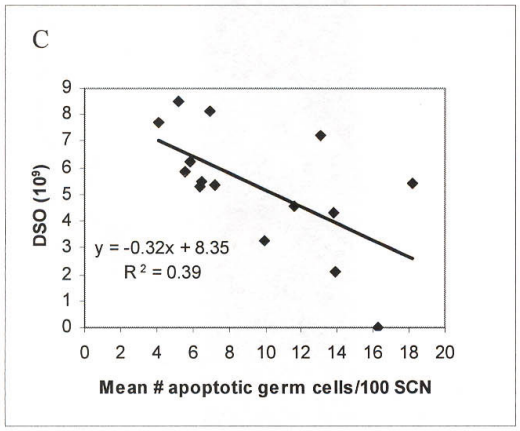
5. Discussion

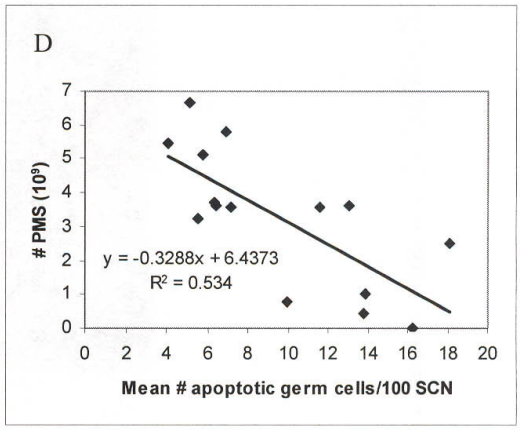
Germ cell apoptotic rates characterizing normal spermatogenesis have previously been established for the stallion [113]. The current study found higher rates of apoptosis in stallions with reduced semen quality. The exact reasons for the increase remain obscure. However, increased germ cell apoptosis is known to occur with conditions such as hormonal imbalance [94], stress [127], viral infection [128], exposure to toxins [76,129-131], and genetic influences (for a review see [6]). Other possible explanations for increased germ cell apoptosis which may relate to spermatogenic dysfunction include the elimination of germ cells with chromosomal abnormalities [107], maintaining an

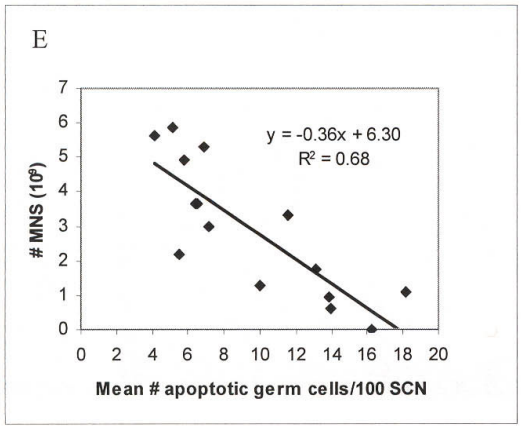
Figure 12. Linear regressions and correlations between germ cell apoptotic rate (number of apoptotic germ cells/100 Sertoli cell nuclei) and daily sperm production per gm of testis (DSP/g), daily sperm production per testis (DSP/testis), daily sperm output (DSO), number of progressively motile sperm in ejaculates at DSO (# PMS), and number of morphologically normal sperm in ejaculates at DSO (#MNS) (Figures A-E, respectively) among 10 stallions with normal semen quality and 7 stallions with reduced semen quality.


A
DSP/G (10 6)
y = -0.53x + 18.50
R 2 = 0.54
Mean # apoptotic germ cells/100 SCN


B
DSP/Testis (10⁹)
y = -0.20x + 4.35
R 2 = 0.77
Mean # apoptotic germ cells/100 SCN


C
DSO (10⁹)
y = -0.32x + 8.35
R 2 = 0.39
Mean # apoptotic germ cells/100 SCN


D
# PMS (10⁹)
y = -0.3288x + 6.4373
R2 = 0.534
Mean # apoptotic germ cells/100 SCN


E
# MNS (10⁹)
y = -0.36x + 6.30
R2 = 0.68
Mean # apoptotic germ cells/100 SCN

optimal germ cell/Sertoli cell ratio [5,108,109], and/or maintaining appropriate architecture and position of germ cells in the seminiferous epithelium [110].

The timing of apoptosis for both groups of stallions coincided with mitotic and meiotic peaks [49], as judged by stage-specific increases in apoptosis. Apoptotic rates were higher in specific stages for stallions with reduced semen quality. While indices for specific germ cell types could not be quantified directly (germ cells lose their characteristic morphology late in the apoptotic process), the increased number of TUNEL-positive germ cells in stages IV and V for stallions with reduced semen quality appeared to be due to increased labeling of spermatogonia (presumably B1 or earlier), late pachytene primary spermatocytes undergoing meiosis, and newly-formed round spermatids. TUNEL-positive round spermatids rarely occurred in stallions with normal semen quality [113]. The observed increases in the number of labeled round spermatids in stallions with reduced semen quality indicate that stallions with reduced semen quality are more prone to losses during spermiogenesis. For the remaining stages VI-VIII, apoptotic germ cells were found most often near the basal lamina (presumably B2 spermatogonia or earlier) but with a lower frequency than occurred in the previous stages IV and V. High numbers of TUNEL-positive round spermatids also contributed to the number of apoptotic germ cells observed in stages VI-VIII.

Decreased DSP per gram of parenchyma (as determined by the number of elongated spermatids in testicular homogenates) in stallions with reduced semen quality has been reported previously [81,85] and is closely related to overall numbers of spermatozoa in ejaculates [132]. Stallions with reduced semen quality had consistently

fewer potential numbers of germ cells per gram and per testis for all germ cell types. Therefore, poor spermatogenic efficiency [105,132] accounted for the low numbers of sperm in the ejaculates of stallions with reduced semen quality. Surprisingly, no difference was detected in the rate of decline in DSP between the two groups of stallions in this study, which differs from results of a previous study in our lab using high and low sperm producing stallions [85]. Possible reasons for this difference include: 1) in our previous report, only those testes producing sperm at greater or less than 1 SD from the mean from a large population of stallions were used as criteria to separate high from low sperm producing stallions; 2) individual stallion variation (i.e. some stallions with reduced semen quality had values for testicular parameters similar to those for normal stallions) (see Table 2).

The greatest impact on germ cell numbers has been shown to occur during spermatocytogenesis and meiosis [105]. This is logical since the loss of a single B1 spermatogonia or pachytene spermatocyte would result in a net loss of 16 and 4 spermatids, respectively. Degeneration during spermatocytogenesis has been demonstrated during the breeding season in the stallion and is reflected by a two-fold increase in the number of A plus B1 spermatogonia, coupled with increased degeneration of B2 spermatogonia [40]. Apoptosis of B spermatogonia in stages IV-VII and declines in spermatogenic potential during the conversion of B1 to B2 spermatogonia suggest that, for the stallion, regulation of spermatogonial proliferation occurs toward the end of spermatocytogenesis (although smaller, immeasurable losses in earlier germ cell types (A spermatogonia) may be just as effective). Additionally,

differences in the potential number of B1 spermatogonia between the two groups of stallions indicate that some germ cell losses had already occurred for stallions with reduced semen quality. Unfortunately, estimating spermatogenic potential in germ cell types earlier than B1 spermatogonia is not possible since the lifespan and number of divisions for type A spermatogonia is not known [40]. Regardless, no degeneration during spermatocytogenesis would result in 60% more germ cells [105], which exceeds the number of germ cells that can be supported by Sertoli cells [39].

The nature of cell death (apoptosis) in elongated spermatids is an intriguing subject currently under much debate. One hypothesis termed “abortive apoptosis” [133] links high apoptotic rates with reduced quality semen suggesting that germ cells initiating apoptosis during spermiogenesis escape removal due to aberrant signal transduction, or by simply overwhelming the phagocytic capacity of the Sertoli cells [134]. Germ cells that fail to be removed then contribute to reduced quality semen in ejaculates. This theory provides a plausible explanation linking germ cell losses during spermiogenesis with reduced semen quality seen in our study. The TUNEL assay does not appear to be useful for evaluating apoptosis in elongated spermatids for any stage, most likely due to the compact nature of the DNA in elongated spermatids and/or the dark staining diaminobenzidine precipitate used for labeling. Future studies examining early apoptotic markers and semen quality are necessary to determine whether apoptosis occurs in elongated spermatids.

Perhaps the most compelling evidence linking apoptosis with reduced quality semen are the significant negative linear relationships apparent between germ cell

apoptotic rate and sperm production, sperm output, or semen quality (Figure 12). High rates of apoptosis effectively decrease the number of germ cells during spermatogenesis, resulting in a lesser number of sperm per gram and per testis, which then translates to lower DSO and fewer normal sperm in an ejaculate. Whether apoptosis is a direct cause of this poor testicular function, or perhaps a secondary response to compromised endocrine and/or paracrine communication remains to be determined.

Endocrine profiles for progressive testicular dysfunction have been described [135] and include decreases in circulating concentrations of inhibin and estradiol with increases in serum FSH concentration. The use of peripheral circulating concentrations of inhibin as an early indicator of declining fertility has been investigated [84]. Stallions with reduced semen quality had lower circulating concentrations of inhibin. Experimental evidence is consistent with the hypothesis that spermatogenesis regulates the level of inhibin secretion [136]. The finding of poor spermatogenic efficiency, as seen for the stallions with reduced semen quality in this study, would agree with normal spermatogenesis being required to maintain adequate concentration of inhibin. Since inhibin is known to be produced by Sertoli cells, inhibin may also have dual roles as a paracrine modulator of germ cell function, as well as endocrine control of FSH secretion in conjunction with estrogen and testosterone [83] for the regulation of spermatogenesis. In men, the measurement of inhibin-B in combination with FSH yielded the highest predictive values for determining fertility status [137]. Concentration of FSH tended to be higher in stallions with reduced semen quality, and suggests that further studies are needed to define the role of inhibin and FSH in stallions with reduced semen quality.

Negative correlations between rates of germ cell apoptosis and circulating estradiol may aid in explaining the increased germ cell apoptosis seen in stallions with reduced semen quality. Estradiol is known to have a sparing effect on germ cell death during spermatogenesis [138] and low concentration of estradiol in the testes might diminish the "protective effect" resulting in higher rates of germ cell apoptosis. The positive correlation between apoptotic rate and FSH concentration agrees with a previous study where meiotic germ cell degeneration correlated with increased serum FSH and decreased DSP during postprophase of meiosis [139]. Higher concentrations of FSH may indicate compromised Sertoli cell function [60], lending further support for the hypothesis that a primary testicular problem exists.

In conclusion, strong links between high rates of apoptosis during specific stages of spermatogenesis and various reproductive parameters (spermatogenic efficiency, circulating hormone concentrations and reduced semen quality) have been established for the stallion. Apoptosis appears to be the primary mechanism for removing germ cells from the seminiferous epithelium during both normal and abnormal spermatogenesis in the stallion, as has been suggested in other species [49,140-143]. Poor spermatogenic efficiency resulting from increased rates of germ cell apoptosis is associated with reduced sperm numbers in the ejaculates of stallions with reduced semen quality. Recent studies demonstrate that prevention of pathologic levels of germ cell apoptosis in the testis is possible in a research setting [130,144,145]. Future studies attempting to improve the semen quality of stallions will likely probe the efficacy of these novel

therapeutic modalities designed to suppress pathologic levels of germ cell apoptosis in the testes.

CHAPTER V

TESTICULAR GERM CELL APOPTOSIS AND FORMATION OF THE SERTOLI CELL BARRIER DURING THE INITIATION OF SPERMATOGENESIS IN PUBERTAL STALLIONS

1. Overview

The regional pattern of seminiferous tubule development in the pubertal stallion was used to study formation of the Sertoli cell barrier (as determined by a vascularly perfused hypertonic-fixative test) and corresponding rates of germ cell apoptosis (TUNEL assay) during the initiation of spermatogenesis. A redefined classification scheme based on lumen score (LS 1 = least developed to LS 7 = mature) was developed to explain the naturally occurring progression of testicular development. Formation of a seminiferous tubule lumen was consistent with events leading to development of a Sertoli cell barrier. Shrinkage artifact (due to the hypertonic-fixative) was observed for all germ cell types and Sertoli cells prior to the formation of a tubule lumen. After the formation of a complete tubule lumen, shrinkage artifact was not observed for germ cells residing within the adluminal compartment indicating the formation of a Sertoli cell barrier. A primary wave of apoptosis (LS 4; $P < 0.05$) appeared to serve as a mechanism for removing early differentiating germ cell types prior to the formation of a tubule lumen, since no advanced germ cell types were found in lumen score 5, thus facilitating both the formation of a tubule lumen, and a Sertoli cell barrier. A second, larger wave of apoptosis (LS 6; $P <0.001$) occurred after the formation of a lumen, but before seminiferous tubule cross-sections contained a full complement of germ cells. Rates of

apoptosis in tubules from pubertal stallions with a full complement of germ cells were similar to rates of apoptosis in testicular parenchyma of adult stallions ($P > 0.05$). The formation of a tubule lumen and development of a Sertoli cell barrier coincide with elevated germ cell apoptotic rates and provide evidence for an integral role of apoptosis during the initiation of spermatogenesis in the pubertal stallion.

2. Introduction

Establishment of spermatogenesis requires a protracted period of time in domestic animals and humans. Initiation of spermatogenesis for most species begins with the conversion of gonocytes into spermatogonia in random, scattered locations throughout the mammalian testis [86] resulting in uniformity of parenchymal coloration or shading [87]. In the stallion, development of spermatogenesis begins centrally and evolves progressively toward the periphery [86,88,89]. A gross-section through a developing testis from a pubertal stallion has light testicular parenchyma with expanding seminiferous tubules in the center, surrounded by dark testicular parenchyma in the periphery, where tubules are less developed. The spermatogenically-inactive, dark tissue gives way to spermatogenically active, light tissue as development progresses [87]. The observable difference in color is attributed to an abundance of fetal Leydig cells and numerous large macrophages (pigment cells) in the interstitium of dark (immature) parenchyma [90].

Seminiferous tubule lumen formation has been used as an indirect marker for establishment of a functional blood-testis barrier [11]. It has also been shown that the

appearance of a tubule lumen within the seminiferous tubules coincides with the development of tight junctions between Sertoli cells of many species [12,146-148]. In pubertal animals, the formation of the small lumina (vacuoles) has been shown to be due to an increase in fluid secretion by the Sertoli cells which occurs in concert with the formation of the Sertoli cell junctional complexes [11,12]. Therefore for the stallion, the formation and aggregation of vacuoles (the beginning of a tubule lumen) may coincide with the formation of a Sertoli cell barrier.

An early physiological apoptotic wave, which occurs among germ cells during the first division and differentiation, has been shown to be necessary for the development of normal, mature spermatogenesis in other species [5,42,91]. The significance of the apoptotic wave during the first round of spermatogenesis has not been elucidated in detail. Studies in mice that inhibit apoptosis by knocking out key apoptotic genes have resulted in accumulation of spermatogonia and spermatocytes and subsequent infertility presumably due to inadequate formation of Sertoli-Sertoli cell junctions [41,42].

The primary objective of this study was to exploit the organized, regional pattern of spermatogenic development in the pubertal stallion to examine the following hypotheses: 1) apoptosis of germ cells occurs at higher rates during specific steps of seminiferous tubule development and 2) development of the Sertoli cell barrier coincides with the formation of a tubule lumen and high germ cell apoptotic rates. A redefined classification scheme describing testicular development in terms of events leading to the

formation of a seminiferous tubule lumen (lumen score) and the presence of advanced germ cell types is described.

3. Materials and Methods

3.1 .Animals and sample collection

To establish developmental patterns in young stallions, testes from 67 normally developing Quarter horse yearling stallions (ages 8-13 months) were harvested during the breeding season by surgical castration in lateral recumbency under general anesthesia (1.0 mg/kg xylazine iv; 2.0 mg/kg ketamine iv). Testes were trimmed of the epididymis, weighed, and sectioned perpendicular to their long-axis, starting from the center to yield samples (approximately 2 mm thick) spanning the entire dimensions of a respective testis. The tunic was partially dissected from the periphery of the testis, leaving a small segment to indicate the dorsal orientation of each testis. Sections were immersion-fixed in 4% paraformaldehyde (Electron Micropscopy Services, Fort Washington, PA), and embedded in paraffin.

Ten testes (from different horses) were chosen for the evaluation of lumen score per unit area, and the number of apoptotic germ cells per unit area within the predominant lumen score (described below). Testis samples were selected (based on testicular weight, development, and completeness of the histological section) to represent the complete range of parenchymal development [all dark (n = 2), mostly dark with some light (n = 3), mostly light with some dark (n = 3), and all light (n = 2)]

observed in these young stallions. Tissue samples from three adult Quarter horse stallions (≥ 5 years) with normal testes size and semen quality (as determined by a previous study in our lab [113]) were used for comparison to the pubertal stallions.

3.2. Lumen scoring system

The progression of seminiferous epithelium development was observed under bright-field microscopy at 400X. Seminiferous tubule cross-sections were classified according to a modification of the lumen scoring system previously reported by Clemmons et al. (1995). Lumen scores (LS) were assigned for each tubule based on the number of germ cell types present (spermatogonia, spermatocytes, round spermatids, and elongated spermatids), the number or aggregation of vacuoles, and the formation of a complete lumen. Tubules were scored on a scale of one (quiescent tubule with no lumen) to seven (complete lumen formation with a full complement of germ cell types) (for a full description see Figure 13) based on the naturally occurring progression of development observed in these young stallions. Digital images were captured (Nikon Alpha Photo® Digital Imager, 2002) at 630 X magnification and processed in Adobe Photoshop® 7.0 to add text and scale bars.

3.3. Hypertonic fixation test

To assess the presence of a Sertoli cell barrier, testes from six yearling stallions (ages 9-14 months) were obtained by surgical castration (as described above). Using

Figure 13: Classification of the equine seminiferous epithelium during the initiation of spermatogenesis based on lumen score (LS 1 to 7). Scale bar represents 40 μm.

Initiation Phase

Lumen score 1: seminiferous tubule contains only gonocytes/spermatogonia and Sertoli cells which are randomly dispersed throughout the tubule. There is no evidence of vacuole formation. Tubule appears quiescent.

Lumen score 2: tubule contains centrally-located gonocytes/spermatogonia and Sertoli cells which have migrated to the basal compartment. A single formed vacuole is present.

Lumen Formation

Lumen score 3: tubule contains gonocytes/spermatogonia and occasional early (leptotene and zygotene) spermatocytes. Several independent, randomly-located vacuoles are present within the seminiferous epithelium.

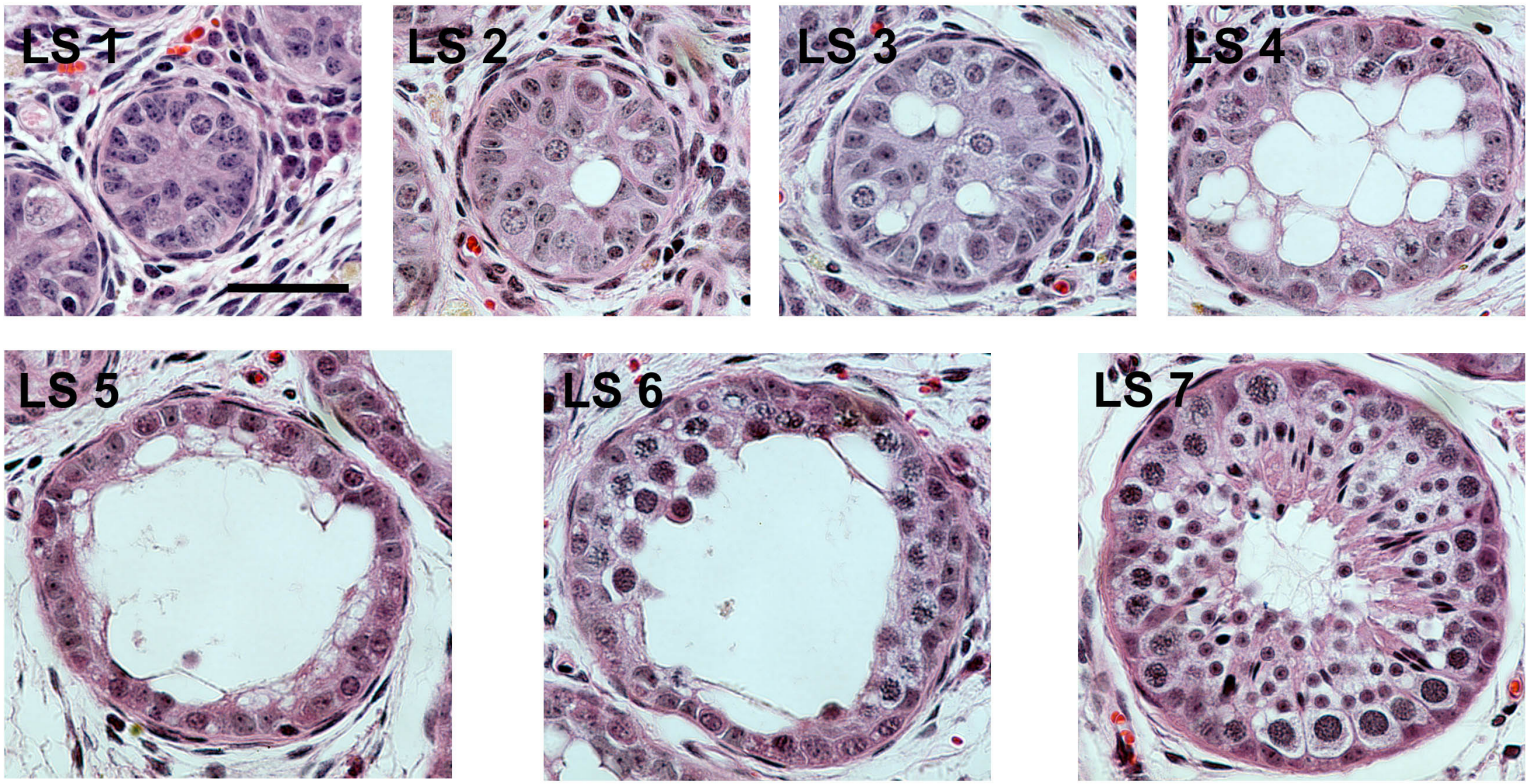
Lumen score 4: tubule contains several connecting/aggregating vacuoles. The most advanced germ cell type is the pachytene spermatocyte.

Lumen score 5: tubule is characterized by an open lumen and a thin (one cell thick) seminiferous epithelium which contains only spermatogonia and Sertoli cells.

Competence

Lumen score 6: tubule is characterized by a complete lumen and an incomplete complement of germ cell types.

Lumen score 7: tubule is characterized by a full complement of germ cells and is capable of classification by the stage of seminiferous epithelium (similar to adult tubules) according to previously reported methods [15,54,113].


LS 1
LS 2
LS 3
LS 4
LS 5
LS 6
LS 7

previously established methods (Russell et al., 1977), testes were perfused via the testicular artery for 30-60 minutes (or until firm) with either 2% glutaraldehyde (450 mOsm/L) (Electron Micropscopy Services, Fort Washington, PA) or 2% glutaraldehyde with 10% dextrose (1214 mOsm/L). Testes were sectioned (as described above) and immersed in their respective fixative for an additional 24 h. Samples were transferred to 0.1M cacodylate buffer and submitted for paraffin embedding. 5-µm sections were mounted on plain glass slides, and counterstained with toluidine blue (1:100 for 2 minutes). The presence/absence of the Sertoli cell barrier was examined in tubules which contain advanced germ cells (primary pachytene spermatocytes or round spermatids since these cell types are expected to reside in the adluminal compartment). Lack of shrinkage artifact for cells in the adluminal compartment in the presence of a vascularly perfused hypertonic fixative indicates the presence of an impermeable barrier (a Sertoli cell barrier) to high concentrations of dextrose. Early spermatocytes (leptotene, zygotene) were not used to evaluate the presence of a barrier due to the uneven distribution of chromatin in the nuclei of these cells and their basal location within the tubule. Less developed tubules (LS 1 and 2) were examined without advanced germ cells, since these tubules only rarely contained advanced germ cell types. Images were captured at 630 X magnification and processed as described above.

3.4. TUNEL assay

Immunohistochemical apoptotic detection of testicular germ cells was performed on 5-µm sections mounted on single or double-width glass slides (depending on testes

size) using the Apoptag® (Intergen Co, Purchase, NY, USA) TUNEL (TdT-mediated dUTP Nick End Labeling) assay according to the manufacturers instructions, previously validated for use in the stallion [113]. Slides were counterstained with periodic acid Schiff (10 minutes) and toluidine blue (1 minute at 1:100 dilution), and fitted with a glass cover-slip.

3.5. Quantification of apoptotic rates and schematic mapping

To quantify apoptotic rates per unit area in the developing stallion testis a grid-overlay plastic film was permanently fixed to each slide. The inside dimensions of each square were approximately 600 μm x 600 μm. The predominant lumen score per unit area (square containing at least 75% of a representative lumen score) and the number of TUNEL-positive germ cells within seminiferous tubules representing the predominant lumen score per square were recorded. Using the data generated by this analysis, representative schematic maps were created to facilitate an understanding of the developmental process for the stallion. The predominant lumen score for each respective square was color-coded using various shades of blue as follows: the darkest blue represents immature, undeveloped parenchyma (LS 1), with progressively lighter shades of blue reflecting progressive seminiferous tubule development and higher lumen scores (LS 2 through 7). Squares with ≥ 3 (empirically determined to account for the low level of apoptosis observed in LS 1 to 3 tubules) apoptotic germ cells per square were identified and shaded red. Numbers within blue or red squares represent the predominant lumen score for that square. Squares containing less than an estimated 25% seminiferous

tubules (interstitial space, vasculature, visceral tunic and mediastinum) are unnumbered and color-coded gray. The number of apoptotic germ cells was not recorded for these squares. An estimated 20% of each testis was not observed due to shading from the grid lines between each square.

3.6. Statistics

Data for the mean number of apoptotic cells per unit area for the predominant lumen score were evaluated by one-way analysis of variance (SAS® Statistical Software; Cary, NC, USA, 2000). Means were separated *post hoc* using the Student-Newman-Keuls test.

4. Results

Testicular weights for the 67 yearling stallions ranged from 8.5 to 68.7 g. At its earliest initiation (testis weight approximately 8-10 grams) spermatogenic development began centrally around the mediastinum and separately, but concurrently in the testicular parenchyma located closest to the epididymis (Figures 14 and 15A). Development was noted to progress both from the mediastinum and the dorsal pole of the testis until all tubules in between the two locations had initiated vacuole/lumen formation. Development then progressed outwards peripherally, creating a “horseshoe-shaped” ring of less developed parenchyma along in the outer distal boundaries (seen in the representative schematic maps of Figures 15B and C). Initiation of tubule development proceeds until no dark parenchyma can be found (testes weight > 50 grams).

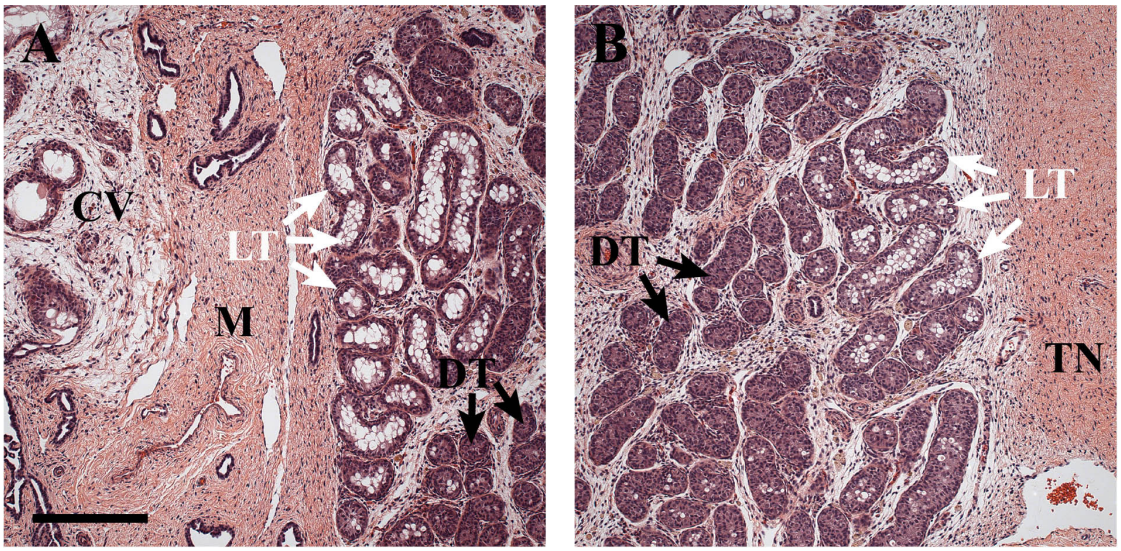


Figure 14: Initiation of spermatogenesis in a single, representative pubertal stallion testis. Panel A depicts tubule development and initial lumen formation in light testicular parenchyma (LT with white arrows) (lumen score 2-4) in central regions of the testis surrounding the mediastinum (M) and central vein (CV). Panel B shows tubule development and lumen formation (within the same testis) beginning separately, and concurrently in the testicular parenchyma proximal to the epididymis. The vaginal tunic (TN) was partially dissected from the periphery of the testis, leaving only a small segment of the tunic to indicate the histological orientation of each testis to indicate the dorsal pole. Spermatogenically inactive, dark tubules (DT with black arrows) (lumen score 1) surround the region of developing tubules in both panels. Scale bar represents 230 μm at 10 X magnification.

The hypertonic fixation test revealed profound shrinkage of all seminiferous tubules, and interstitial cells (Leydig and macrophage). Within cross-sections of seminiferous tubules, shrinkage of every cell type (germ cells and Sertoli cells) was evident for lumen scores 1 through 4 (Figure 16). In lumen score 4 tubules (the first lumen score with pachytene spermatocytes), shrinkage artifact was detected in pachytene

Figure 15: Schematic color-coded maps representing pubertal development in stallion testis. The predominant lumen score for each respective square (600 μm^2) is represented by various shades of blue. The darkest blue represents immature, spermatogenically inactive parenchyma (dark tubules), with lighter shades of blue representing progressive seminiferous tubule development (light tubules) and a higher lumen score (identified by the number in the square). Squares with ≥ 3 apoptotic germ cells per square (empirically determined) were shaded red. Squares containing less than an estimated 25% seminiferous tubules [interstitial space, vasculature, visceral tunic indicating histological orientation at the dorsal pole of the testis* and mediastinum (M)] are unnumbered, and color-coded gray. The number of apoptotic germ cells was not recorded for these squares. Panel A (9.2 g testis) demonstrates the initiation of spermatogenesis centrally near the mediastinum, and separately, but concurrently in the parenchyma near the dorsal pole of the testis (arrow). Panel B (18.4 g testis) represents regional, progressive tubule development starting from the center, towards the periphery with a horse-shoe shaped ring of less developed tubules (lumen scores 1 and 2) in the outer margins. Panel C (52.2 g testis) demonstrates progressive, regional seminiferous tubule development and regionally higher apoptotic rates in the center of the testis (apoptotic wave) (lumen scores 6 and 7) surrounding the mediastinum.

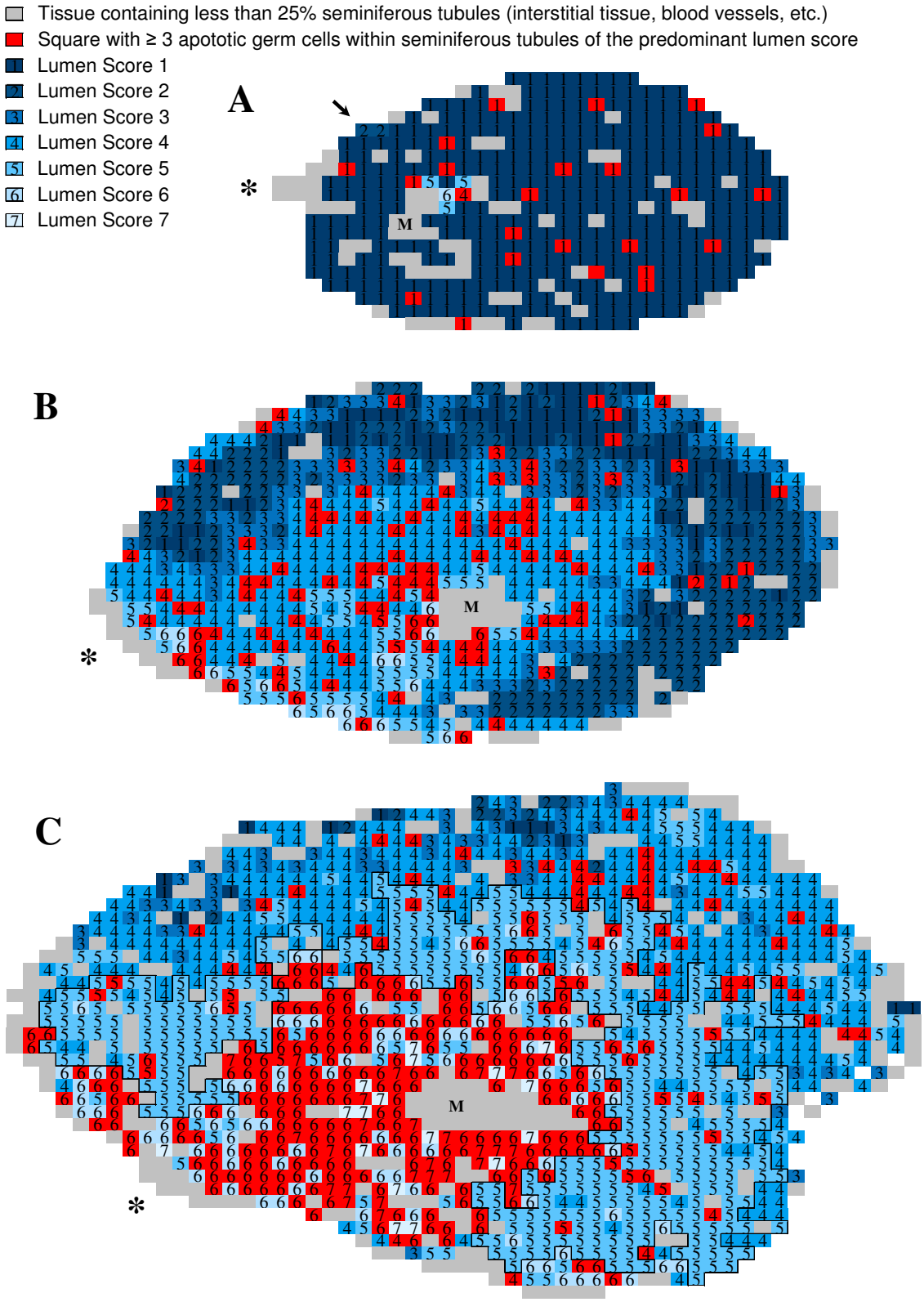
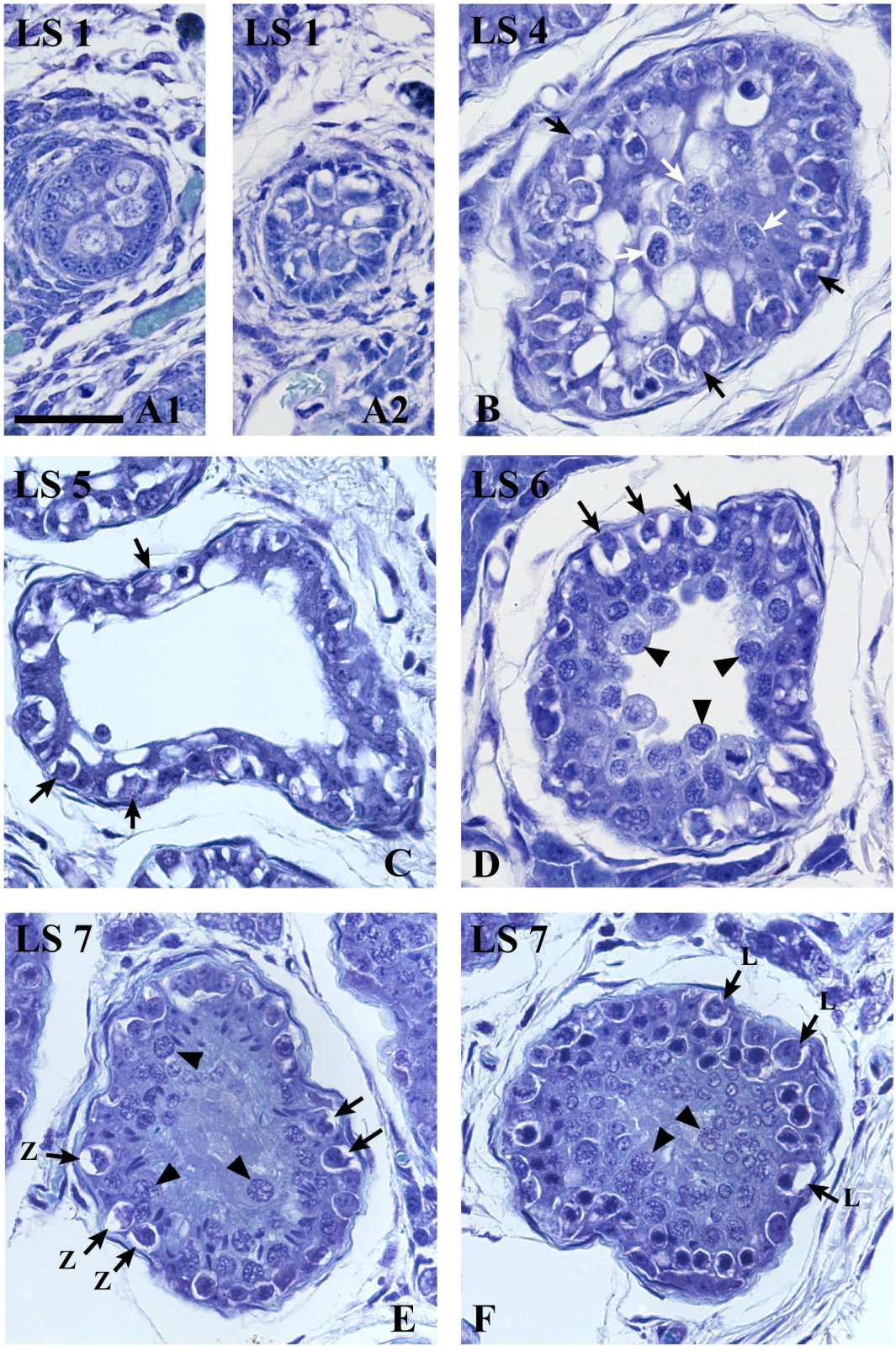

Tissue containing less than 25% seminiferous tubules (interstitial tissue, blood vessels, etc.)
Square with ≥ 3 apototic germ cells within seminiferous tubules of the predominant lumen score
Lumen Score 1
Lumen Score 2
Lumen Score 3
Lumen Score 4
Lumen Score 5
Lumen Score 6
Lumen Score 7
A
B
C
M
M
M
*
*
*

Figure 16: Hypertonic fixation test according to lumen score. Marked shrinkage of all seminiferous tubules occurred due to the hypertonic fixative. Panels A1 and A2 demonstrate the effects of either 2% glutaraldehyde or 2% glutaraldehyde with 10% dextrose (hypertonic fixative) on lumen score 1 tubules. All other control tubules were similar to tubules in figure 1. Exaggerated shrinkage artifact (arrows) due to the hypertonic fixative can be observed for all germ cells and Sertoli cells within the seminiferous epithelium of lumen score 1 through 5 as shown in panels A2, B, and C indicating that an impermeable barrier (Sertoli cell barrier) to high concentration of dextrose had yet to be formed. Panel B depicts a lumen score 4 tubule with shrinkage artifact surrounding advanced germ cells (white arrows) and the lack of a Sertoli cell barrier. Panel D demonstrates effects of the same hypertonic fixative on lumen score 6 tubules. Marked shrinkage artifact (arrows) occurred in the basal compartment of the seminiferous tubule, while no shrinkage artifact was observed for advanced germ cells (arrowheads; pachytene spermatocytes) in the adluminal compartment indicating the presence of an intact Sertoli cell barrier. Panels E and F represent lumen score 7 tubules with a full complement of germ cells (stages IV and I, respectively) and an intact Sertoli cell barrier indicated by shrinkage artifact in the basal compartment including leptotene (L) and zygotene (Z) spermatocytes. Scale bar represents 40 μm.


LS 1
A1
LS 1
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F

spermatocytes indicating that an impermeable barrier to high concentrations of dextrose had not been formed. Lumen score 5 tubules demonstrated shrinkage artifact for both spermatogonia and Sertoli cells, but assessment of the Sertoli cell barrier was not possible since advanced germ cell types are not present in this lumen score. In lumen score 6 tubules (the first lumen score with round spermatids), advanced germ cells (pachytene spermatocytes and round spermatids) in the adluminal compartment showed no signs of shrinkage artifact. The most advanced tubules (LS 7) in pubertal stallions with a full complement of germ cells, demonstrated pronounced shrinkage artifact for germ cells [spermatogonia as well as preleptotene, leptotene (Figure 16E) and zygotene (Figure 16F) spermatocytes] in the basal compartment only.

A small, primary wave of apoptosis occurred prior to the development of a complete lumen. Lumen score 4 tubules had a higher mean number of apoptotic cells within the predominant lumen score per unit area ($P < 0.05$) than lumen scores 1-3 or lumen score 5 tubules (Figure 17A). A second, larger apoptotic wave occurred after the formation of a complete lumen. Lumen score 6 tubules had a higher number of apoptotic cells per unit area than all other lumen score tubules ($P < 0.001$). The rate of germ cell apoptosis in lumen score 7 tubules was similar to the level found in adult seminiferous tubules ($P > 0.05$).

Schematic, color-coded maps were generated as a result of recording the predominant lumen score for each square, and the number of TUNEL-positive germ cells within the predominant lumen score for each square (Figure 15). Waves of regional development corresponding to lumen score (shades of blue) are evident. A similar

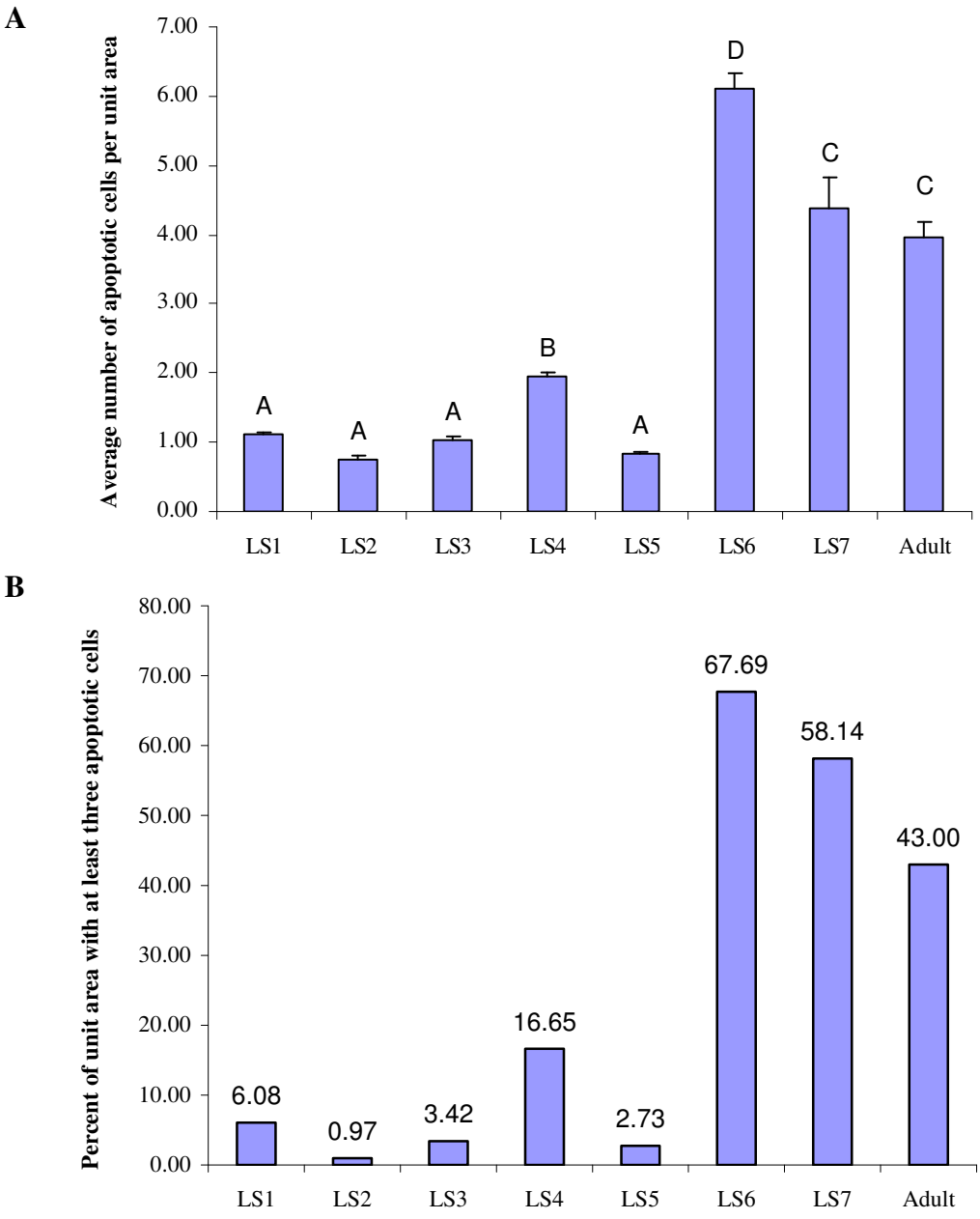

Figure 17: Mean number of apoptotic germ cells per unit area (A) and the percentage of unit area with at least 3 apoptotic germ cells (B) according to the predominant lumen score within a square for yearling (n = 10) and adult (n = 3) stallions.
^{A,B,C,D} Means with different superscripts are different ($P < 0.05$).

relationship was seen for the percentage of unit area with at least three apoptotic cells and lumen score when compared to the mean number of apoptotic germ cells (Figure 17B). Squares containing ≥ 3 apoptotic germ cells per unit area (flagged red) completely surround the mediastinum (grey unnumbered squares in the central region) and indicates a regionally higher apoptotic rate for lumen scores 6 and 7 when compared to less mature parenchyma in the periphery.

5. Discussion

Gross differences in shading of pubertal equine testis have been documented as early as the turn of the century [88]. Nishikawa and Horie [89] reported that gross coloration of equine testicular parenchyma parallels tubule development and testicular size in pre-pubertal horses. These authors also reported that spermatozoa were first detected in centrally located tubules followed by a uniform presence of these spermatids in most tubules throughout the testis. Our study confirms these findings, and also demonstrates that initiation occurs separately, and concurrently in the testicular parenchyma located proximal to the epididymis (Figure 14). This finding lends further support for localized control of the initiation of spermatogenesis [10] though the exact mechanism remains to be defined. Speculation on the nature of the mechanism suggests localized changes in vasculature [147,149], paracrine/endocrine signaling, and/or patterns of gene expression.

Testicular development in the stallion begins with an initiation phase and marks the transition from a semi-quiescent (semi is implied since rare instances of

differentiating germ cells and TUNEL-positive germ cells were observed even in the most immature tissue) lumen score 1 tubule with Sertoli cells and gonocytes randomly located throughout the seminiferous epithelium. Differentiating germ cells were found in increasing numbers during early tubule development (lumen score 1 through 4), prior to the formation of a Sertoli cell barrier (as judged by the hypertonic fixation test). Supporting these results, one previous study found that spermatogenesis can proceed in the absence of a Sertoli cell barrier up to, and including the DNA synthesis phase of spermatocytes without triggering an immune response [150]. Collectively, these results suggest that initial differentiation of germ cells occurs prior to, or perhaps stimulates the formation of a Sertoli cell barrier, but development to round spermatids was never observed before lumen score 6.

The lumen formation phase begins with the development of a single vacuole (LS 2). Small vacuoles increase in number (LS 3) and then aggregate (LS 4) to initiate the development of a tubule lumen. Fluid secretion alone, even without the presence of a barrier, has been suggested to be responsible for the formation of very small lumina [12]. Testicular perfusion with a hypertonic fixative caused cytoplasmic and nuclear shrinkage artifact in conjunction with exaggerated extracellular space throughout the seminiferous epithelium of all early tubules (LS 1 through LS 5), indicating that a permeability barrier (Sertoli cell barrier) to high concentrations of dextrose had yet to be formed. Therefore, in the stallion, formation of small vacuoles in early lumen score tubules is likely due to secretion of fluids by Sertoli cells prior to the formation of a Sertoli cell barrier.

The final process in lumen formation results in a dramatic increase in tubule lumen diameter (lumen score 5) coupled with a reduction in seminiferous epithelium height (a single cell layer). One previous study in the mouse noted that a gradual increase in lumen diameter occurred prior to formation of the Sertoli cell barrier, but that more than 90% of the increase took place after the Sertoli cell barrier was formed reflecting an increased tightness of the Sertoli cell barrier, increased fluid secretion by Sertoli cells, and/or remodeling of structural elements with increased tubule size [12]. Ligation of efferent ducts has also resulted in increased tubular fluid pressure and a dramatically increased lumen diameter in a relatively short time [151-153]. Applying this information to the developmental pattern observed in the pubertal stallion would imply that the Sertoli cell barrier is formed early in lumen score 5 when the contact between Sertoli cells is maximized.

The hypertonic fixation test is an effective method for determining the initial formation of a permeability barrier but is not a sensitive method for determining the degree of tightness between the junctions [12]. This test only detects the presence or absence of a Sertoli cell barrier and future studies should consider the use of exclusion properties of molecules to compare with current results. Advanced germ cells in the adluminal compartment of lumen score 4 demonstrated shrinkage artifact, but this was not observed for advanced germ cells in the adluminal compartment of lumen score 6. It should then be considered that formation of the Sertoli cell barrier occurs in lumen score 5, in coinciding with the increase in lumen diameter. Results of this study also demonstrate that lumen score 5 tubules can be found throughout the range of testicular

weights used in this study. Therefore, for the stallion, the Sertoli cell barrier appears to develop regionally, over time (many months), rather than asynchronously along the length of a tubule [154], or by stage of seminiferous epithelium [91,155,156] as suggested in other species. Additional studies are required to elucidate the dynamics of the Sertoli cell barrier and tubule lumen formation in the stallion.

An early physiological apoptotic wave, which occurs among germ cells during the first division and differentiation, has been shown to be necessary for the development of spermatogenesis in other species [5,42,91]. The significance of the apoptotic wave during the first round of spermatogenesis has not been established. The magnitude of the first increase in the number of apoptotic cells (in lumen score 4 tubules) was minimal, though statistically significant. Previous estimates of the number of germ cells removed by this primary wave of apoptosis are greater than 80% [5]. For our study, the increased apoptotic rate in the early wave arises from the degeneration of a small number of germ cells engaged in spermatogenesis prior to the formation of a tubule lumen and a Sertoli cell barrier. Germ cells that enter spermatogenesis during lumen scores 1 through 4 must be removed from the developing seminiferous epithelium, since no differentiated germ cells are present in lumen score 5 tubules. Therefore, it can be suggested from our study that during the initial differentiation of germ cells, apoptosis serves as an important mechanism to remove these germ cells from the seminiferous epithelium. Once germ cells have been removed, Sertoli cell contact is maximized so that attachment/organization of junctional proteins [157] and the establishment of a functional barrier can ensue.

The second increase in number of apoptotic cells in lumen score 6 tubules (Figure 17) is three-fold larger than the first and corresponds to removal of the first germ cells that could potentially complete spermatogenesis. This “second initiation” of spermatogenesis after lumen score 5 consists of several consecutive cycles of the seminiferous epithelium, each developing further than the previous cycle. The extraordinarily high losses of germ cells (above that for normal, mature spermatogenesis) may be attributed to a gaining of “competence” perhaps associated with the complexity of producing haploid sperm cells. During pubertal development in the stallion, the number of germ cells have been shown to dramatically increase, while the number of Sertoli cells per gram decreases [87]. Apoptosis would be a logical mechanism establishing a critical germ cell:Sertoli cell ratio beyond the capacity of a single Sertoli cell [42]. Another explanation for the dramatic increase in apoptosis might be the absence of survival signals secreted by round spermatids [158]. Although round spermatids were found in lumen score 6, their numbers may be too few for effective signal propagation.

In summary, the present study provides new insight into pubertal development and the role of apoptosis during the initiation of equine spermatogenesis. Establishing a detailed pattern of development will allow future studies to more closely examine treatment effects (i.e. toxins, glucocorticoid therapy) on spermatogenic development and the formation of a Sertoli cell barrier. Initial germ cell differentiation precedes the formation of a Sertoli cell barrier. However, formation of the barrier coincides with the development of a tubule lumen. Higher rates of germ cell apoptosis occurred during

specific steps of seminiferous tubule development which can be viewed on schematic maps, illustrating regional waves of apoptotic germ cells in the development of pubertal stallion testis. Future studies should exploit the unequivocal pattern of development in the stallion for a better understanding of the events leading to the initiation of spermatogenesis.

CHAPTER VI

CONCLUSIONS

In conclusion, germ cell degeneration in the stallion appears to be mediated by apoptosis. Apoptosis appears to be the primary mechanism for removing germ cells from the seminiferous epithelium during both normal (stages IV, V and VI) and abnormal (stages IV, V, VI and VIII) spermatogenesis in the stallion. The TUNEL assay is a valuable tool for profiling levels of germ cell apoptosis during spermatogenesis and use of the assay is warranted by the assay's reliability and wide berth of acceptance in the scientific community. The TUNEL assay has been shown to be particularly useful as a means of establishing the harmful effects of drugs or toxins on spermatogenesis. While use of the TUNEL assay to quantify levels of germ cell death in the testis is a valuable tool, the assay is not without limitations.

To date, most research utilizes orchiectomy as the primary method for obtaining sufficient testicular parenchyma (tissue samples > $1cm^3$ in our studies) which facilitates the evaluation of hundreds of seminiferous tubule cross-sections. Future studies should investigate less invasive protocols such as testicular biopsies, or fine-needle aspiration cytology, although each of these methods has their own set of limitations (i.e. fewer seminiferous tubule cross-sections and loss of tubule architecture, respectively). A less invasive protocol for harvesting tissue samples would be useful clinically, and more practical for use in valuable stallions.

Another research avenue that remains to be pursued is the use of the TUNEL assay to quantify apoptosis in elongated spermatids and/or spermatozoa. Our studies did

not detect labeled elongated spermatids or spermatozoa. One explanation would be the inability to correctly identify labeled apoptotic cells due to the dark brown DAB precipitate on top of a highly compact, dark staining nucleus. Florescent antibodies are commercially available for use with this assay, and may prove to be better for identification of apoptosis in these cell types. Apoptosis that occurs in spermatozoa (within the testis, epididymis, and even ejaculates) is a potential avenue for future development of clinical tests which might explain a stallion's fertility status.

The TUNEL assay is also limited by its ability to detect germ cell apoptosis late in the apoptotic process, which largely overlooks the pathological process that may result in increased levels of germ cell death. Additionally, subtle changes in the rate of germ cell death likely precede the clinical diagnosis of reduced semen quality (low numbers of morphologically normal sperm) which may not be detected by the TUNEL assay. Future efforts should incorporate other related molecular markers (i.e. Fas-FasL), gene expression patterns and/or hormone concentrations (circulating and local) in conjunction with TUNEL analysis. Future research may also reveal novel molecular markers (i.e. proteins, enzymes, or antibodies) detectable in peripheral blood samples. Concentrations of these novel markers may relate to increased testicular germ cell apoptosis and provide a diagnosis which explains the hypospermatogenic state of a stallion.

Another research avenue that may be pursued in hopes of developing a treatment for idiopathic hypospermatogenesis includes the treatment of affected stallions with apoptosis inhibiting drugs. Several recent studies demonstrate that prevention of

pathologic levels of germ cell apoptosis in the testis is possible in a research setting [130,144,145]. Future studies attempting to improve the semen quality of stallions may probe the efficacy of these novel therapeutic modalities which appear to suppress pathologic levels of germ cell apoptosis. However, pursuit of these novel treatments should always bear in mind that apoptosis (especially in testicular germ cells) is a double-edged sword. Since apoptosis is necessary for normal spermatogenesis, pharmacologic inhibition of pathways that eliminate cells with DNA damage may result in the propagation of transformed cells leading to tumorigenesis and germ-line transmission of genetic defects [6]. This line of research should aim to "prevent (or rescue) apoptotic cells", and these compounds should always be tested extensively on experimental animals *in vitro* prior to any application *in vivo*.

Poor spermatogenic efficiency resulting from increased rates of germ cell apoptosis is associated with reduced sperm numbers in the ejaculates of stallions with reduced semen quality. Currently, methods to evaluate potential daily sperm production (DSP) involve extensive histomorphometric procedures. These studies are generally expensive, time-consuming (months to years) and require cautious interpretation between studies, even when produced in the same lab. Implementation of new technology using computer-assisted morphometry has the potential to greatly accelerate up the process, especially concerning the determination of volume density. Currently, a field viewed under the microscope (point-counting) is evaluated, data recorded and then the evaluator moves to a new field. Software on computers designed to perform image analysis can save fields for future analysis/reanalysis, perform calculations set by the

researcher, aid in elimination of wish bias, and analyze data statistically. Enticing as these new technologies may be, their use must first be rigorously tested against decades of validation of previous methods. Carefully conducted studies should evaluate the strengths and weaknesses to determine the technology's reliability.

New insight into pubertal development and the role of apoptosis during the initiation of equine spermatogenesis has been established Future studies may use the detailed pattern of development to more closely examine treatment effects (i.e. toxins, glucocorticoid therapy) on spermatogenic development. Additionally, studies examining gene expression patterns can be designed to elucidate specific genes responsible for upregulating apoptotic pathways during the initiation of spermatogenesis, with hope that the knowledge gained from these experiments can be applied clinically to stallions with idiopathic subfertility. Future studies should exploit the unique pattern of spermatogenic development in the stallion for a better understanding of spermatogenesis in mammals as a whole.

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APPENDIX A

PROTOCOL FOR THE APOPTAG IN SITU HYBRIDIZATION ASSAY

Materials Provided in kit:
Equilibration Buffer
Reaction Buffer
TdT Enzyme
Stop/Wash Buffer
Anti-Digoxigenin-Peroxidase
Plastic Coverslips (throw away- DO NOT USE!!!)

Materials Not Supplied with kit:

1. Distilled water
2. Hydrogen peroxide, 30%
3. PBS (50 mM sodium phosphate, pH 7.4, 200 mM NaC1)
4. 0.05% (w:v) diaminobenzidine (DAB) in staining buffer (PBS or other staining buffer recommended by manufacturer). Freshly prepare and store in the dark. Filter immediately before use, then add 0.02% (v:v) hydrogen peroxide.
5. Slide mounting medium (Permount or equivalent)
6. Counterstain (see Appendix B)
7. 100% ethanol
8. Xylene
9. Ethanol: absolute, 95%, 90%, 80%, 70%, diluted in distilled water
10. Proteinase K, 20 g/ml in PBS.
11. 4% paraformaldehyde (PAF)
12. Incubator at 37^{o}C
13. Adjustable micropipettors
14. Glass or plastic coplin staining jars (100 mL or 500 mL)
15. Humidified chamber
16. Glass coverslips
17. Light microscope

Making Reagents for Apoptag® Protocol

1. PBS

For 1L PBS 50mM Sodium Phosphate, 200 mM NaCl pH 7.4
FW NaCl = 58.44g X 200M = 11688g/L
For a 200mM divide by 1000 = 11.68g NaCl
FW Sodium Phosphate Monobasic = 141.96g X 50M = 7098g/L
For a 50mM divide by 1000 = 7.098g Sodium Phosphate

2. Proteinase K

Need 20µg/ml

$\frac{20\mu g/ml}{20{,}000\mu g/ml} = 0.001$ or 1µl/ml PBS

3. Dnase I

Need 20µg/ml

$\frac{20\mu g/ml}{20{,}000\mu g/ml} = 0.001$ or 1µl/ml PBS

4. DAB

0.05g/100ml or .2g/400ml PBS

$\frac{0.03\%}{30\%}$ *X 400 =.4 ml or 400 ul H2O2*

Apoptag Protocol for Paraffin Embedded Samples

All steps performed at room temperature unless otherwise stated

I. Deparaffinization protocol (steps a-e)

1) Insert PAF fixed, paraffin embedded glass slides into an appropriate slide rack w/ wire handle
2) Set up 8 sequential coplin staining jars (100 mL or 500mL depending on 3 of slides) and fill with respective solution until the fluid level just covers the slides when immersed.

a. Xylene (2 times, 5 minutes) to remove the paraffin (check slides to ensure adhesion to slide)
b. 100% ETOH (2 times, 5 minutes) to start rehydration process
c. 95% ETOH (1 time, 5 minutes)
d. 70% ETOH (1 time, 5 minutes)
e. 1X PBS (2 times, 5 minutes) to finish rehydration

II. Proteinase K treatment

3) Immerse slides in Proteinase K, 20µg/ml (as described above) for 15 minutes using a coplin staining jar.
4) Wash with 1X PBS (2 times, 5 minutes) in a coplin staining jar.

III DNase positive control

5) Prepare positive control using DNase I (20µg/ml as described above) for 15 minutes using rat regressing mammary slides.
6) Wash with 1X PBS (2 times, 5 minutes) in a coplin staining jar

IV. Quench endogenous peroxidases

7) Quench with 2% H_2O_2 in 1X PBS (1 times, 5 minute) using a coplin staining jar.
8) Wash with 1X PBS (2 times, 5 minutes) in a coplin staining jar.
9) Prepare tissue withequilibration buffer (15-30 minutes) directly from bottle using 60-70µl per tissue section.

V. TdT enzyme

10) Apply TdT enzyme (77µl Reaction Buffer: 33µl TdT Enzyme) directly on slide using 60- 70µl per tissue section. Incubate slides for 1 hour in a humidity chamber at 37°C. Omit TdT enzyme for negative control slide.
11) Stop/Wash buffer (34 ml Distilled H_20:1 ml Stop/Wash buffer) using a coplin staining jar (1 time, 10 minutes).

12) Wash with 1X PBS (2 times, 5 minutes) in a coplin staining jar.

VI. Antibody labeling

13) Apply anti-digoxigenin antibody supplied with the kit directly on slide for 30 minutes .

14) Wash with 1X PBS (3 times, 5 minutes) in a coplin staining jar. Do not skip this step since any excess/unbound antibody needs to be removed.

VII. DAB development

15) Prepare DAB solution as described above (0.05% DAB) in a glass coplin staining jar Develop for 8-10 minutes watching closely for color development. A single slide can periodically be checked under the microscope to identify labeled cells. Handle DAB with gloves and dispose in labeled waste container

16) Rinse with distilled H_20 (3 times, 5 minutes) in a coplin staining jar.

VIII. Counterstaining, dehydration, and coverslips

17) Counterstain using the PAS/Toluidine blue protocol outlined in Appendix C.

18) Rinse in distilled H_20 (3 times, 5 minutes) and dehydrate tissue with:

a. 70% ETOH (1 time, 5 minutes)
b. 95% ETOH (1 time, 5 minutes)
c. 100% ETOH (2 times, 5 minutes)
d. Toluene (1 time, 5 minutes)

19) Apply glass coverslip using Permount media

APPENDIX B

EXTENDED CRITERIA FOR STAGING THE EQUINE

SEMINIFEROUS EPITHELIUM

Stage I

Composed of preleptotene and leptotene as well as pachytene primary spermatocytes. Preleptotene nuclei are generally in final stages and are primarily localized to stage VIII. Leptotene spermatocyte nuclei have uneven heterochromatin distribution due to initial stages of DNA synthesis. Sb1 round spermatids are always present and well rounded with smooth nuclear appearance and well formed caps. Developing tails may be seen in plastic sections with good morphology. All Sd2 spermatids (the most mature) have been released into the tubule lumen by spermiation.

Stage II

Only leptotene and pachytene primary spermatocytes are seen with leptotene closer to the basement membrane and uneven chromatin distribution. Pachytene nuclei are larger with evenly dispersed, but clumping heterochromatin. The round spermatids have begun initial elongation stages and have lost their round appearance. Tails are evident and orientation of the acrosome begins to point towards the basal membrane.

Stage III

Two generations of primary spermatocytes with the identification of zygotene spermatocytes is possible along the basement membrane with uneven heterochromatin that appears to be very unevenly distributed leaving a clear side of the nuclei. Pachytene spermatocytes are large and preparing to begin meiosis. Sc spermatids have their elongated shape, but chromatin compation does not appear as dense. (The shortest stage of spermatogenesis and therefore the least frequent)

Stage IV

One of the most recognizable stages due to length of stage and frequency with which it occurs. Zygotene spermatocytes line the basal compartment and pachytene spermatocytes are extremely large with some exhibiting metaphase plates of meiotic division. Secondary spermatocytes are evident with a slightly larger, smoother nucleus than a typical round spermatid. Sc spermatids are evident with slightly more chromatin condensation.

Stage V

Newly formed pachytene spermatocytes line the basal compartment of the tubule. Newly formed round spermatids with spherical nuclei and a smooth nuclear appearance have completed meiosis. No meiotic figures should be present. A few round spermatids are visible which contain acrosomic vesicles. The golgi apparatus is frequently seen in these newly formed round spermatids supplying the formation of the acrosome. Sd1 spermatids are packed in tight clusters and

remain deep within the crypts of the Sertoli cell. Chromatin is tightly condensed and packaged in these cells.

Stage VI

Stage VI marks the appearance of B spermatogonia with clumps of nuclear chromatin at the nuclear margins. Nuclei appear round or slightly oval and slightly darker that their A spermatogonia counterparts and are always in contact with the basement membrane. Sa round spermatids all have some presence of the acrosomal vesicle/cap and the golgi apparatus is still often observed in close contact with the acrosome. Sd1 spermatids begin to migrate towards the lumen and are not as tightly clustered as previous stages.

Stage VII

Exactly the same cell types as Stage VI except that acrosomal caps have progressed farther along nuclear margins for the Sa round spermatids. The appearance of the Sd2 elongated spermatids mark the final stage of differentiation for the mature spermatozoa. Sd2 elongated spermatids have nearly reached the lumen.

Stage VIII

The final stage in spermatogenesis. B spermatogonia are no longer present and have divided to form the preleptotene primary spermatocyte. These cells at first glance resemble B spermatogonia, but a closer look reveals that preleptotene spermatocyte nuclei are slightly smaller than B spermatogonia nuclei. Preleptotene spermatocytes are lined up across the basal membrane and begin to push the pachytene spermatocytes away from the basal membrane. Preleptotenes start to resemble chromatin patterns of the leptotene phase. Sa spermatids are fully round with developed acrosomes. Spermiation is occurring as fully differentiated spermatozoa are released into the lumen for transport to the epididymis for additional maturation. Residual bodies build up at the luminal border to be phagocytized by the Sertoli cell.

APPENDIX C

PROTOCOL FOR COUNTERSTAINS

Toluidine Blue

(1) 2% stock Toluidine Blue
2.0 grams in 100 ml distilled water

(2) 2% stock Sodium Borate Buffer
2.0 grams in 100 ml distilled water

(3) Add Together
25 ml Toluidine Blue stock
25 ml Sodium Borate stock

(4) Filter

(5) Place in 10 cc syringe with a millipore filter (acrodisc).
Filled by needle aspiration.

PAS Toluidine Blue Stain

Counterstain for apoptag assay on Equine Testes
(courtesy of Sarah Jones)

1) Slides received in water (distilled)
2) 5 minutes in 0.5% Periodic Acid
3) Rinse well in water (end up in distilled)
4) 10 minutes in Schiffs Reagent
5) 10 minute rinse in running tap water
6) Rinse in distilled
7) 2 minutes in Toluidine Blue Stain 1:100
8) Rinse in water
9) 2 quick dips in 95% x 2
(Must be quick in the 95% because the stain washes out, Abs not as critical)
10) 2-3 quick dips in Abs x 2
11) 10-15 dips in xylene until clear of alcohol
12) 10-15 dips in Pro-Par x 4
13) Coverslip

Toluidine Blue Stain 1:100
5 ml of 7 Blue (1% in 1% borax
500 ml of 1% borax

APPENDIX D

PROTOCOL FOR FIXATIVES

Preparation of 2% glutaraldehyde in 0.1 M Cacodylate buffer

(Recipe from Larry Johnson's Lab)

Materials:
1) 100 ml or 200 ml plastic fixative bottle.
2) Two (2) 10cc syringes with needles attached.
3) Two (2) 100 m1 graduated cylinders.
4) Ampule of 70% glutaraldehyde.
5) Stock solution of 0.2 M sodium Cacodylate.

Procedure for 2 ml ampule of 70 % of glutaraldehyde (Makes 70 mls of fixative)

1) Using graduated cylinder, add 35 mls of 0.2 M sodium Cacodylate buffer and 28 m1s of distilled water into the 150 m1 fixative bottle.
2) Fill a 10cc syringe with 5 mls (5cc) of distilled water.
3) Fill another 10 cc syringe with air.
4) Take a scored glutaraldehyde ampule and by applying pressure to the top of the ampule pop off the top of the ampule.
5) Take the ampule of glutaraldehyde and begin pouring it into the fixative bottle. Usually the glutaraldehyde will not readily pour out (especially when it has been refrigerated) due to a vacuum in the ampule. Therefore, take the syringe of air and inject it up into the ampule as you pour the glutaraldehyde into the fixative bottle. The air being injected into ampule will displace the glutaraldehyde, forcing it into the fixative bottle.
6) After emptying the ampule, position the ampu1e right side up, inject the 5cc of distilled water from the other syringe into ampule in order to rinse the remaining glutaraldehyde from the ampule. Gently swirl the water in the ampule, and then pour the 5cc of water from the ampule into the fixative bottle.

Procedure for 5 ml ampule of 70% glutaraldehyde (Makes 175 mls of fixative)

1) Using graduated cylinder, add 87.5 mls of 0.2 M sodium Cacodylate buffer and 77.5 m1s of distilled water into the 150 m1 fixative bottle.
2) Fill a 10cc syringe with 5 mls (5cc) of distilled water.
3) Fill another 10 cc syringe with air.
4) Take a scored glutaraldehyde ampule and by applying pressure to the top of the ampule pop off the top of the ampule.

5) Take the ampule of glutaraldehyde and begin pouring it into the fixative bottle. Usually the glutaraldehyde will not readily pour out (especially when it has been refrigerated) due to a vacuum in the ampule. Therefore, take the syringe of air and inject it up into the ampule as you pour the glutaraldehyde into the fixative bottle. The air being injected into ampule will displace the glutaraldehyde, forcing it into the fixative bottle.
6) After emptying the ampule, position the ampu1e right side up, inject the 5cc of distilled water from the other syringe into ampule in order to rinse the remaining glutaraldehyde from the ampule. Gently swirl the water in the ampule, and then pour the 5cc of water from the ampule into the fixative bottle.

Preparation of 0.2 M Cacodylate buffer (400ml batch)

Materials:
1) Sodium Cacodylate (Molecular Weight = 214.02)
2) 12 M Hydrochloric acid (HCL)
3) Distilled water

Glassware:
1) 600 m1 beaker
2) 100 ml graduated cylinder
3) 500 m1 graduated cylinder
4) 20 ml beaker
5) 1 m1 pipette
6) 10 ml pipette

Procedure:

1) Dissolve 17.42 grams of sodium Cacodylate in 100 ml of distilled water in the 600 ml beaker.
2) Pipette 5.5 ml of distilled water into the 20 ml beaker and add 0.5 ml of 12 M HCL.
Mix and transfer 5.0 mls into the 600 ml beaker containing the dissolved sodium Cacodylate.
3) Add 295 mls of distilled water to the 600 ml beaker. mix thoroughly, and withdraw 1.0 ml for pH check: (using a clean Pasteur pipette).
Pour the solution into the sodium Cacodylate Stock Bottle and record the pH on the bottle. The pH should be between 7.2 and 7.4

Preparation of 0.2 M Cacodylate buffer (800ml batch)

(Recipe from Larry Johnson's lab)

Materials: 1) Sodium Cacodylate (Molecular Weight = 214.02)
2) 12 M Hydrochloric acid (HCL)
3) Distilled water

Glassware: 1) 1000 m1 beaker
2) 250 ml graduated cylinder
3) 500 m1 graduated cylinder
4) 20 ml beaker
5) 1 m1 pipette
6) 10 ml pipette

Procedure:

1) Dissolve 34.24 grams of sodium Cacodylate in 200 ml of distilled water in the 1000 ml beaker.
2) Pipette 11 ml of distilled water into the 20 ml beaker and add 1 ml of 12 M HCL. Mix and transfer 10 mls into the 1000 ml beaker containing the dissolved sodium Cacodylate.
3) Add 590 mls of distilled water to the 1000 ml beaker. mix thoroughly, and withdraw 1.0 ml for pH check: (using a clean Pasteur pipette).
Pour the solution into the sodium Cacodylate Stock Bottle and record the pH on the bottle. The pH should be between 7.2 and 7.4

Preparation of 4% paraformaldehyde

(recipe from Dr Ing's lab)

For 200 ml solution
Heat 100 ml distilled H20 to 60^{o}C
Add 8 g paraformaldehyde (granular)
Add 1-2 drops of 2M NaOH, until solution turns clear
Remove from heat
Add 100 ml of 2x PBS
Bring final volume up to 200 ml with distilled water

APPENDIX E

TISSUE PROCESSING PROTOCOL FOR EPON EMBEDDING

General Outline: (reprinted from Larry Johnson's lab)

NOTE: If the tissue to be embedded is being stored in glutaraldehyde, transfer the tissue into 0.1 M cacodylate buffer the night before processing. Use gloves during all procedures.

I. 0.1 M Cacodylate buffer	(3 rinses)	5 minutes each
II. 1% Osmium in 0.1 M Cacodylate Buffer (DO THIS UNDER THE HOOD)		1-4 hours (refrig.)
III. 0.1 M Sodium Acetate	(3 rinses)	5 minutes each
IV. 0.5% Uranyl Acetate		Overnight (refrig.)
V. 0.1 M Sodium Acetate	(3 rinses)	5 minutes each
VI. Dehydration		
50% ethyl alcohol	(keep cold)	5 minutes
70% ethyl alcohol	(keep cold)	10 minutes
95% ethyl alcohol	(keep cold)	10 minutes
100% ethyl alcohol	(keep cold)	15 minutes
100% ethyl alcohol	(room temperature)	20 minutes
100 % ethyl alcohol	(room temperature)	1 hour
VII. Make Epon/Araldite Resin		
VIII Propylene Oxide (wear gloves)	(2 rinses) (room temp.--under hood)	15 minutes each
IX. 50% Propylene Oxide - 50% Epon mixture (wear gloves and rotate samples)		3-4 hours
X. 100% Epon/Araldite Resin (wear gloves and rotate samples)		Overnight

Xl. Embed tissue in EM bullets with Epon resin.

XII. Heat embedded tissue in oven at 60 degrees Celsius, for 8-12 hours.

Specific Steps:

I. Three (3) rinses with 0.1 M Cacodylate Buffer **Time: 5 minutes each**

Materials: 1) 0.2 M cacodylate buffer
2) distilled water

Glassware: 1) graduated cylinder
2) beaker
3) pasteur pipettes and bulbs

Preparation: of 0.1 M cacodylate buffer

Stock solution of 0.2 M cacoclylate buffer will be diluted 1:1 with distilled water. Using a clean graduated cylinder, measure out an appropriate volume of 0.2 M cacodylate buffer and transfer to a clean beaker. Use the same graduated cylinder, measure out an equal volume of distilled water and transfer to the same beaker. Mix well.

Procedure:

Use pasteur pipettes to change solution in tissue vials. Pipette off the solution bathing the tissue and discard it. Using a fresh pipette, replace the solution with fresh 0.1 M cacodylate buffer. Let the solution bathe the tissue for at least 5 minutes (longer than 5 minutes is permissible). Swirl vials every 1-2 minutes. Repeat this procedure 3 times.

II. Fixation with Osmium (work in hood) **Time: 1-4 hrs in dark**
(in the refrigerator) 1% $0s0_4$ (osmium) in 0.1 M cacodylate buffer

*****Osmium is VERY DANGEROUS. Only work with it under the FUME HOOD *****

Materials: 1) 2% $0s0_4$
2) 0.2 M cacodylate buffer

Glassware: 1) 2 small beakers or vials
2) sterile disposable pipette
3) pasteur pipettes and bulbs

Preparation: of 1% $0s0_4$ in 0.1 M cacodylate buffer

2% $0s0_4$ stock solution will be diluted 1:1 with 0.2 M cacodylate buffer. — Use a sterile disposable pipette of an appropriate volume for the number of vials being processed. Being careful not to touch the working end of the stock bottle of Osmium, pipette an appropriate volume of 2% $0s0_4$ from the stock bottle and transfer to a small beaker or vial. This is the only occasion when a pipette may be used to draw directly from the stock bottle. Pour an appropriate volume of 0.2 M cacodylate buffer into a clean beaker. Using

the same disposable pipette, draw from the beaker an equal amount of buffer and transfer to beaker or vial containing osmium. Discard any excess cacodylate buffer. Mix osmium and buffer well.

Procedure:

After solution in vials has been changed to Osmium/buffer solution, exposure of vials to light should be kept to a minimum as light will reduce 0s04. It is best to store osmicating tissue in the dark (i.e. refrigerator) as soon as possible. If this osmium fixation must be carried out at room temperature, place vials in a dark lined box in a drawer.

III. Three (3) rinses with 0.1 M Sodium Acetate **Time: 5 minutes each**

Materials: 1) 0.1 M sodium acetate

Glassware: 1) beaker
2) pasteur pipettes and bulbs

Procedure:

Use pasteur pipettes to change solution in tissue vials. Pipette off the solution bathing the tissue with a pipette and discard. Using a fresh pipette, replace the solution with fresh 0.1 M sodium acetate. Let the sodium acetate bathe the tissue for at least 5 minutes (longer than 5 minutes is permissible). Swirl vials every 1-2 minutes. Repeat this procedure 3 times.

IV. Overnight in 0.5% Uranyl Acetate (under Fume Hood) (slightly radioactive)

Materials: 1) 0.5% Uranyl Acetate

Glassware: 1) beaker
2) pasteur pipettes and bulbs

Procedure:

Pipette off the 0.1 M Sodium Acetate bathing the tissue, and replace with 0.5% Uranyl Acetate solution. Changing of solutions in vials should be done as quickly as possible and vials stored in refrigerator overnight to minimize reaction of Uranyl Acetate with light. DO NOT expose the stock solution of Uranyl Acetate to light for an extended period of time. Place tissue in refrigerator overnight.

III. Three (3) rinses with 0.1 M Sodium Acetate **Time: 5 minutes each**

Materials: 1) 0.1 M sodium acetate

Glassware: 1) beaker
2) pasteur pipettes and bulbs

Procedure: Use same procedure as in Step III

VI. Dehydration

Materials:	1) 50% ethyl alcohol	(cold)
	2) 70% ethyl alcohol	(cold)
	3) 95% ethyl alcohol	(cold)
	4)100% ethyl alcohol	(cold)
	5) 100% ethyl alcohol	(room temperature)

Glassware: 1) beakers
2) pasteur pipettes and bulbs

Procedure:

Rinse in ice, not refrigerator:	50% ethyl alcohol	for	5 minutes
	70% ethyl alcohol	for	10 minutes
	95% ethyl alcohol	for	10 minutes
	100% ethyl alcohol	for	15 minutes
Rinse in room temp.alcohol:	100% ethyl alcohol	for	20 minutes
	100% ethyl alcohol	for	20 minutes

VI. Make epon/araldite resin

Materials: 1) DDSA
2) Embed 812
3) Araldite 502
4) DMP-30
5) 4 plastic pipettes
6) Tri-pour beaker
7) Wooden applicator sticks

The formula used to calculate the total amount of resin needed is:

(# vials of tissue x 3) + # pieces of tissue to embed = total amount of resin needed in mis.

Procedure:

Determine the total amount of resin needed using the above formula, round that number up to the nearest multiple often, and use a top-loading balance to make that amount of epon using one of the below recipes. Mix all chemicals in the order as shown (i.e. DDSA, E812, A502, and then DMP-30).

	For 10 mls	For 20 mls	For 30 mls	For 40 mls
DDSA	5.303	10.605	15.908	21.211
E812	2.750	5.500	8.250	11.000
(yields)	8.053	16.105	24.158	32.211
A502	1.755	3.509	5.264	7.019
(yields)	9.808	19.164	29.422	39.230
DMP-30	0.193	0.386	0.578	0.771
(yields)	10.01	20.000	30.000	40.001

VIII. Two (2) rinses with Propylene Oxi4 Time: 15 minutes each

Materials: 1) propylene oxide

Glassware: 1) beaker (oven-dried before using)
2) pasteur pipettes and bulbs

General Considerations:

Propylene oxide is kept at room temperature. The bottle should be tightly capped as propylene oxide will absorb moisture from the air.

Procedure:

Using a clean beaker, oven-dried prior to use, pour out an appropriate volume of propylene oxide and begin pipetting. Discard any excess propylene oxide and return beaker to oven between changes.

IX: 50:50 propylene oxide: Epon resin Time: 3-4 hours

Materials: 1) propylene oxide
2) epon resin
3) plastic tri-pour beaker
4) syringe
5) pasteur pipettes and bulbs

Preparation: of 50:50 propylene oxide mixture:

Using a syringe, mix Epon resin 1:1 with propylene oxide in a plastic beaker. The final amount of 50:50 propylene oxide-Epon resin should equal

imi of mixture per vial of tissue. Samples should be rotated on a rotating device for the 3-4 hour time duration.

Procedure:

Extract and discard the existing propylene oxide bathing the tissue in the vials, and add about 1 ml of 50:50 propylene oxide-Epon mixture.

X. Pure 100% Epon resin **Time: Overnight**

Materials:
1) Epon resin
2) pasteur pipettes and bulbs
3) plastic pipette with tip cut off

Procedure:

Using a pasteur pipette, decant the 50:50 propylene oxide - Epon mixture from vials, and add about imi of pure 100% Epon resin. Samples should be rotated on a rotating device overnight.

XI: Embed tissue in Pure 100% Epon

Materials:
1) EM bullets
2) Epon resin
3) plastic pipettes with tip cut off
4) flat tipped applicator sticks

Procedure:

Place bullet labels inside the EM bullets. Place 1 drop of epon in each EM bullet. Using a flat- tipped applicator stick, gently remove each piece of tissue from vial and place in appropriately labeled EM bullet with a flat side of the tissue block gently pressed against the floor of the EM bullet which will allow greater ease of trimming and sectioning later on. After placing tissue in EM bullet, fill the EM bullet with 100% epon.

XII.: Bake the Epon bullets

Procedure:

Place the EM bullets in the oven and bake at 60 degrees Celsius, for 8-12 hours using the automatic timer attached to the oven.

NOTE: The formula used to determine the amount of Epon resin to make in step no. VII. was derived from the following considerations:

Epon resin needed for 1) the 1:1 mixture of resin with propylene oxide in step VIII.
2) the 100% resin in step IX.
3) the embedding of tissue in bullets instep X.

Respectively.

- the amount of resin needed for 50% propylene oxidel50% resin = # vials x 1 ml
- the amount of resin needed for 100% pure epon = # vials x 2 mls
- the amount of resin needed for embedding tissue in bullets = # tissue pieces x l ml

Thus, the formula used to calculate the total amount of resin needed for these three steps is:

(# vials x 3) + # pieces of tissue to embed = total amount of resin needed in mls.

General Considerations:

- ALWAYS WEAR GLOVES!! If any of the chemicals are dripped or spilled, you can clean them up with 95% ethanol.

- The resin will not be completely mixed until it sits for at least 20 minutes before you use it. It may LOOK mixed thoroughly, but molecularly it is not. It must sit for at least 20 minutes after mixing.

- Mix the chemicals in the order listed. Check to make sure that it is the correct chemical by reading the label.

Procedure:

- Place a tri-pour beaker on the large top-loading balance. Tare it.

- Pour or pipette the chemicals into the beaker. Pipette very slowly as you near the desired weight.

Use a new pipette with each chemical.

- Add each chemical in the same way until all have been added.

- Take three or four wooden sticks and slowly stir the chemicals until there is no longer a visible separation between them.

- After mixing, place the beaker of resin into a vacuum-dessicator. This step is vital to the embedding process!! Pull a vacuum on the resin until the majority of the air bubbles have collected at the top of the beaker. This usually takes 10-15 minutes. Stay with the resin and watch to time it properly. Too much vacuum will cause the resin to bubble even more.

- Allow the resin to stay under the vacuum until you are ready to use it. This will keep water in the air from contaminating the resin. Each time you release the vacuum to remove some of the resin, be sure to check that the resin is still mixed well. If it is not, then stir it and vacuum it again before you use it.

- After each removal of resin, pull a vacuum again on the remaining resin. You must protect the resin from water contamination whenever possible.

- When the embedding process is finished, the unused resin may be cooked (polymerized) in the oven, and discarded in the trash.

Preparation of 2% Osmium (Work in hood)

Materials: 1) ampule of 0s04 (0.5 or 1.0 gram size)
2) distilled water

Glassware: 1) 2% Osmium Stock Bottle
2) graduated cylinder

Procedure:
1) Get ampule of Osmium Stock Bottle
2) Wash and rinse ampule thoroughly; dry the ampule with kimwipe so that no fingerprints contaminate the glass.
3) Drop the clean ampule into the 2% Osmium Stock Bottle.
4) Place the stopper on the Osmium Stock Bottle, and shake it until the ampule breaks.
5) Add an appropriate volume of distilled water to make a 2% solution (25 mls H20 for a 0.5 g ampule, or 50 mls H20 for a 1.0 g ampule).
6) Shake and leave in refrigerator at least 24 hours before using.

Preparation of 0.1 M Sodium Acetate (400 ml batch)

Materials: 1) Sodium Acetate (Molecular Weight = 136.08)
2) Distilled water
Glassware: 1) 600m1 beaker
2) 500ml graduated cylinder
Procedure:
1) Dissolve 5.44 grams of Sodium Acetate into 400 mis of distilled water. Mix thoroughly.
2) Pour solution in Sodium Acetate Stock Bottle.

Preparation of 0.5% Uranyl Acetate (100 ml batch)

Materials:
1) Uranyl Acetate (Molecular Weight = 424.15)
2) Distilled water

Glassware:
1) 500 ml beaker
2)100 ml graduated cylinder

Procedure:
1) Dissolve 0.5 grams of Uranyl Acetate into 100 mis of distilled water. Mix thoroughly.
2) Pour solution in Uranyl Acetate Stock Bottle.

VITA

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